REMARKS

Claims 1-3, 6-20, 23, 26-29 and 32-45 were pending in this application. Claims 1, 15, 17, 18 and 27 were amended. Thus claims 1-3, 6, 10-20, 23, 26-29 and 32 are still pending in the present application and under consideration. Applicant maintains that the amendments do not introduce any new matter.

Declaration

A new Declaration in compliance with 37 CFR 1.67(a) is submitted herewith as requested by the Examiner.

Objection to the Amended Disclosure under 35 USC 132(a)

The amendments filed 11/7/05 to Table 2 were objected to by the Examiner under 35 USC 132(a) for introducing new matter. The Examiner has previously pointed out a discrepancy in Table 2, i.e., reciting the same number in two columns of the Table. Applicant maintains that the presence of the same number in two columns of Table 2 is indicative of a typographical error. Based on the Examiner's remarks, Applicant went back to the NIH grant that was filed concurrently with the instant patent application and was based on the same data. Applicant corrected Table 2 of the instant specification, using numbers provided in the NIH grant application (page 15). Applicants is attaching a copy of the NIH grant application for Examiner's review. Accordingly, the Examiner is kindly requested to withdraw this objection.

Rejection under 35 U.S.C. 112, Second Paragraph

The Examiner rejected claims 1, 15, 17, 18 and 27 under 35 USC 112, second paragraph for being indefinite for failing to particularly point out and distinctly claim the subject matter. Which applicants regard as the invention.

In response, Applicant has amended claims 1, 17 and 27 to properly state the Markush group. In addition, claim 15 was amended to recite "said at least one β-lactam antibiotic," which is a term that has an antecedent basis in step (c) claim 13. Claim 18 was also amended in order to end with a period. Accordingly, the Examiner is kindly requested to withdraw these rejections.

Rejection under 35 U.S.C. 112, First Paragraph

The Examiner claims 1-3, 6, 10-20, 23, 26-29 and 32 are rejected under 35 USC 112, first paragraph, enablement requirement. The Examiner's alleges that the specification does not enable one of ordinary skill in the art to make catalytic antibodies that can attach a label to a molecule (1) due to the lack of working examples and (2) because, in Examiner's opinion, the art of catalytic antibodies, unlike the art of monoclonal antibodies, is unpredictable.

Applicant respectfully traverses. First, working examples are not required to satisfy the enablement requirement (MPEP Section 2164.02). As admitted by the Examiner, the Specification provides extensive disclosure of the methods of making and using catalytic antibodies, as well as target molecules to be modifies by disclosed antibodies. Every stage of the process is disclosed in a great detail therefore enabling a person of ordinary skill in the art to practice the claimed invention without undue experimentation. The disclosure also teaches how to test the antibodies for the desired activity, e.g., catalytic antibody can be identified by screening human phage antibody display libraries against an antibiotic-target conjugate. The specification teaches selecting labels that exhibit a low but detectable reaction with the desired target in the absence of a catalyst, for example, the conjugation

reaction of β-lactam antibiotics with proteins (Specification, page 9, line 15 – page 10, line 10). The same passage in the specification also notes that the fact that the uncatatalyzed reaction can occur at a slow rate places a lower burden on the catalyst and may only require that the catalyst bind to both the target and label so as to hold them in close proximity and increase their effective concentrations. In addition, the specification is not limited to selection of catalytic antibodies by panning phages and also teaches a variety of other approaches including directed evolution under selective pressure and/or the mutation of catalysts with similar chemical activities but different structural specificity. The fact that the specification does not provide working examples of the elicitation of catalytic antibodies does not support the Examiner's rejection.

The Examiner fails to explain why the methodology taught in the specification would not enable one of ordinary skill in the art to practice the claimed invention. As such, the Examiner's arguments are without merit and the pending claims should be found enabled in satisfaction of 35 U.S.C. § 112, ¶ 1. *E.g.*, *Marzocchi*, 439 F.2d at 224, 169 U.S.P.Q. at 369-70; *Pishevar*, 2002 WL 1801082, at *4-*5; *Dow*, 1997 WL 33116047, at *2.

Applicants submit that one of ordinary skill in the art would be able to practice the presently claimed subject matter in view of the specification and the prior art without undue experimentation. The test for enablement is not whether any experimentation is necessary, but whether, if experimentation is necessary, it is undue. *In re Angstadt*, 190 U.S.P.Q. 214 (CCPA 1976). See also, MPEP § 2164.01. The fact that experimentation may be complex does not necessarily make it undue if those skilled in the art typically engage in such experimentation. *In re Certain Limited - Charge Cell Culture Microcarriers*, 221 U.S.P.Q. 1165, 1174 (Int'l Trade Comm'n 1983); *M.I.T. v. A.B. Fortia*, 227 U.S.P.Q. 428 (Fed. Cir. 1985); *In re Wands*, 8 U.S.P.Q.2d 1400 (Fed. Cir. 1988). See also, MPEP § 2164.01.

Contrary to the Examiner's suggestion, the specification need not provide examples or specific description of embodiments for the entire scope of the invention. Detailed procedures for making and using an invention may not be necessary if the description of the invention itself is sufficient to permit those skilled in the art to make and use the invention [MPEP §2164]. A patent does not teach, **and preferably omits**, what is well known in the art. *In re Buchner*, 18 U.S.P.Q.2d 1331, 1332 (Fed. Cir. 1991); *Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 231 U.S.P.Q. 81, 94 (Fed. Cir. 1986), *cert. denied*, 480 U.S. 947 (1987); *Lindemann Maschinenfabrik GMBH v. American Hoist & Derrick Co.*, 221 U.S.P.Q. 481, 489 (Fed. Cir. 1984). [See also, MPEP § 2164.01].

Second, the Examiner's reliance on Janda et al., Schultz, Sinha and Yu to support the allegation of unpredictability is misplaced. While citing Janda et al., Schultz, Sinha and Yu the Examiner also implicitly admits that at least one catalytic antibody was obtained in each of the cited references, showing that appropriate antibody activity is achieved despite any inherent variability associated with generating catalytic antibodies (or antibodies in general). The Examiner, describes the experimental effect observed in Janda et al., but fails to mention that Janda et al. explain the effect and further teaches that "From this findings an approach is suggested to improve antibody catalytic efficiency." (Janda et al, page 2504). Yu et al and Schultz (two studies coming from the same research group) teaches that "Both this study and recent work by Reymond et al. show that haptens containing a positive charge corresponding to the anomeric center of a cyclic acetal can elicit antibodies with hydrolytic activity." (Yu et al., page 340, right-hand column). Finally, although Taffik et al. suggests that the rates achieved to date were "modest," Taffik et al. does review a large number of examples of successful generation of catalytic antibodies.

Applicants urge that the state of the relevant art is high. The following two publications reflect the high state of the relevant art. Copies of the references are enclosed for the Examiner's convenience as supporting reference material.

(1) Nevinsky GA, Semenov DV, Buneva VN.

Catalytic antibodies (Abzymes) Induced by Stable Transition-State Analogs

Biochemistry (Moscow) 2000; 65(11): 1233-44.

The Nevinsky reference includes twenty-four examples compiled in a comprehensive table format of successful catalytic antibodies productions where transition-state analogs were employed. This review further demonstrates that in view of the high state of relevant art, the applicants had possession of the instant invention.

(2) Stevenson JD and Tomas NR.

Catalytic antibodies and other biomimetic catalysts

Nat. Prod. Rep. 2000; 17: 535-577.

The Stevenson reference is a comprehensive review which provides multiple examples of the successful use of various transition state analogs in eliciting catalytic antibodies for both ester and amide hydrolysis (chapters 2.6 and 2.7).

Applicant urges that the claims are fully enabled by the disclosure in the Specification and further in view of the high state of relevant art. Although the specification discloses an embodiment in which a β-lactam antibiotic is attached to a target molecule, the teachings of the specification are considerably broader. The section of the specification describing "labels" for modifying target molecules (Specification, page 8, line 22 – page, line 10) lists a variety of suitable labels for use with the methods of the invention and also describes properties of the labels that can be used to select for other suitable labels.

The Examiner specifically alleges that the Specification is not enabling with respect to on-naturally occurring enzyme (Claims 17-20, 23 and 26).

Applicants respectfully traverse. The specification clearly discloses the applicability of all methods to "Catalysts of biological origin such as enzymes. . ." (Specification, page 7, line 25). The specification goes further to note that "Catalytic antibodies are especially preferred catalysts." (Specification, page 8, line 3). Every method disclosed in the specification in great detail is applicable to naturally occurring enzymes that are modified as disclosed in the specification (thus, resulting in non-naturally occurring enzymes). Even further, while the Examiner alleges that the art of catalytic antibodies is unpredictable, the Examiner makes no such argument in relation the field of enzyme modification. A number of companies successfully practice in this area of technology, the list including, but not limited to, Direvo AG (http://www.direvo.com/), Diversa Corp. (http://www.diversa.com/) and Maxygen (http://www.maxygen.com/nopage.php).

The Examiner fails to explain why the methodology taught in the specification would not enable one of ordinary skill in the art to practice the claimed invention. As such, the Examiner's arguments are without merit and the pending claims should be found enabled in satisfaction of 35 U.S.C. § 112, ¶ 1. *E.g.*, *Marzocchi*, 439 F.2d at 224, 169 U.S.P.Q. at 369-70; *Pishevar*, 2002 WL 1801082, at *4-*5; *Dow*, 1997 WL 33116047, at *2.

Thus, one of ordinary skill in the art would readily recognize from the original disclosure that the present invention is enabled. Therefore, Applicant requests that this rejection be withdrawn.

Claims 1-3, 6, 10-20, 23, 26-29 and 32 were rejected under 35 USC 112, first paragraph, written description requirement. (Office Action, pages 6-7).

In response, Applicant respectfully traverses. Applicant submits that the function of the written description requirement is to ensure that a patent is granted to inventors who had possession, as of the filing date of the application relied on, of the specific subject matter later claimed by them; how the

specification accomplishes this is not material. *In re Smith*, 178 U.S.P.Q. 620 (CCPA 1973). Therefore, the test for written description under 35 U.S.C. §112, first paragraph, is whether the originally filed specification reasonably conveys to a person having ordinary skill that Applicants had possession of the subject matter later claimed. *In re Kaslow*, 217 U.S.P.Q. 1089 (Fed. Cir. 1983). [See also, MPEP, Section 2163.02].

The Specification provides extensive disclosure of the methods of making and using catalytic antibodies, as well as target molecules to be modifies by disclosed antibodies. Every stage of the process is disclosed in a great detail therefore enabling a person of ordinary skill in the art to practice the claimed invention without undue experimentation. The disclosure also teaches how to test the antibodies for the desired activity, e.g., catalytic antibody can be identified by screening human phage antibody display libraries against an antibiotic-target conjugate. The specification teaches selecting labels that exhibit a low but detectable reaction with the desired target in the absence of a catalyst, for example, the conjugation reaction of β-lactam antibiotics with proteins (Specification, page 9, line 15 – page 10, line 10). The same passage in the specification also notes that the fact that the uncatatalyzed reaction can occur at a slow rate places a lower burden on the catalyst and may only require that the catalyst bind to both the target and label so as to hold them in close proximity and increase their effective concentrations. In addition, the specification is not limited to selection of catalytic antibodies by panning phages and also teaches a variety of other approaches including directed evolution under selective pressure and/or the mutation of catalysts with similar chemical activities but different structural specificity. The Specification also makes it clear that the disclosure applies to non-naturally occurring enzymes. (Specification, page 7, line 25)

Thus, one of ordinary skill in the art would readily recognize from the original disclosure that Applicants invented the presently claimed subject matter. Applicants submit that the Examiner's

allegation that the specification is deficient in that it does not show working is not relevant to a determination of whether Appellants' have satisfied the written description requirement of the first paragraph of 35 USC 112. Therefore, Appellants request that this rejection be reversed.

In view of the foregoing, it is respectfully submitted that this application is now in condition to be allowed and the early issuance of a Notice of Allowance is respectfully solicited. If there are any issues or amendments the Examiner wishes to discuss, the Examiner is encouraged to contact the undersigned.

Respectfully submitted,
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I. BIOGRAPHICAL SKETCHES AND BIBLIOGRAPHY

Mark T. Martin, Ph.D. will be the Principal Investigator. Dr. Martin is Principal Scientist (1997-) and Director of the Discovery Research Group (1993-) at IGEN International, Inc. His research interests in this capacity include catalytic antibodies, electrochemiluminescence, carbon nanotube chemistry, and molecular imprinting. He was the Director of Enzymology at the company from 1990 until 1993. From 1989 until 1990, he consulted for IGEN in the field of Catalytic Antibodies. During the period of 1989-1990, he was also an Assistant Professor of Biochemistry at North Carolina State University. Before that, Dr. Martin was a postdoctoral fellow at Oxford University (enzyme kinetics) and Harvard Medical School (biochemistry and biophysics). He received a Ph.D. degree in Biochemistry for the University of North Dakota. Dr. Martin was a pioneer in catalytic antibody enzymology. He has published numerous journal articles and holds several patents in the fields of catalytic antibodies, phage display, and electrochemiluminescence. Some of these are listed below:

1) Blackburn, G.F. et al. (1990) *Potentiometric biosensor employing catalytic antibodies as the molecular recognition element.* Anal. Chem. 62, 2211.
2) Martin, M.T. et al. (1990) *Mechanistic characterization of a catalytic antibody.* in Catalytic Antibodies, Ciba Found. Symp., Wiley, Chichester, UK, pp. 188-197.
3) Martin, M.T. et al., (1991) *Mechanistic characterization of a tyrosine-dependent catalytic antibody.* Biochemistry 30, 9757.
4) Angeles, T.S. et al. (1993) *Isoabzymes: structurally and mechanistically similar catalytic antibodies.* Biochemistry 32, 12128.
5) Angeles, T.S. & Martin, M.T. (1993) *Mechanism-based catalytic antibody inactivation.* Biochem. Biophys. Res. Commun. 197, 696.
6) Titmas, R.C. et al. (1994) *Aspects of antibody-catalyzed primary amide hydrolysis.* Appl. Biochem. Biophys. 47, 277.
7) Martin, M.T. et al. (1994) *Antibody-catalyzed hydrolysis of an unsubstituted amide.* J. Am. Chem. Soc. 116, 6508.
8) Smith, R.G. et al. (1995) *Cloning and bacterial expression of an esterolytic sFv.* in Methods in Molecular Biology, Vol. 51: Antibody Engineering Protocols, S. Paul ed., Humana, Totowa, NJ.
9) Dong, L.D. & Martin, M.T. (1996) *Enzyme-triggered formation of electrochemiluminescent ruthenium complexes.* Anal. Biochem. 236, 344.
10) Jameison, F. et al. (1996) *Electrochemiluminescence-based quantitation of classical clinical chemistry analytes.* Anal. Chem. 68, 1298.
11) Liang, P. et al. (1996) *Electrochemiluminescence-based detection of β-lactam antibiotics and β-lactamases.* Anal. Chem. 14, 2426.
12) Liang, P. et al. (1996) *Light emission from ruthenium-labeled penicillins signaling their hydrolysis by β-lactamase.* J. Am. Chem. Soc. 118, 9198.
13) Martin, M.T. (1996) *Commercially valuable catalytic antibodies: the life to come.* Drug Discovery Today 1, 187.

- Martin, M.T. et al. (1997) *Reaction-based selection for expression and concentration of catalytic moieties.* U.S. Patent 5,631,137.
- Liang, P. et al. (1997) *Electrochemiluminescent monitoring of compounds.* U.S. Patent 5,643,713.
- Martin, M.T. (1997) *Electrochemiluminescence assay.* U.S. Patent 5,641,623.
- Martin, M.T. & Dong, L. (1998) *Electrochemiluminescent enzyme assay.* U.S. Patent 5,804,400.
- Smith, R.G. et al. (1999) *Isolation of catalytic antibodies using phage technology.* U.S. Patent 5,855,885.
- Massey, R.C. et al. (1999) *Graphitic nanotubes in luminescence assays.* U.S. Patent 5,866,434.
- Martin, M.T. et al. (1999) *Reaction-based selection for expression and concentration of catalytic moieties.* U.S. Patent 5,891,648.
- Napper, A. et al. (1999) *Catalytic antibodies which hydrolyze primary amides and methods for eliciting such antibodies.* U.S. Patent 5,900,237.

Dr. Richard J. Massey will be a Senior Advisor on the project. Dr. Massey is a founder of IGEN International, Inc., and has been President and COO of the company since February 1992, a Director of the company since 1990, and served as Vice President from 1985 to 1992. From 1981 until he joined IGEN in 1983, Dr. Massey was a faculty member in the Microbiology and Immunology Department at Rush Medical Center in Chicago. Prior to that, he was Senior Research Scientist at the National Cancer Institute, Frederick Cancer Research Center. Dr. Massey received a Ph.D. in virology from the University of Illinois. Dr.

Massey can be considered a co-inventor of catalytic antibodies since he holds the earliest and broadest patents in the field. He also holds numerous other patents in catAb and ECL technology. A partial list is shown below:

- Powell, M.J. et al. (1999) *Transition state analogs.* U.S. Patent 5,952,462.
- Paul, S. et al. (1997) *Catalytic antibody components.* U.S. Patent 5,658,753.
- Schochetman, G. & Massey, R.J. (1997) *Methods of catalyzing chemical reactions.* U.S. Patent 5,658,753.
- Schochetman, G. & Massey, R.J. (1992) *Catalytic antibodies.* U.S. Patent 5,156,965.
- Schochetman, G. & Massey, R.J. (1991) *Method for producing antibodies which catalyze chemical reactions.* U.S. Patent 5,037,750.
- Schochetman, G. & Massey, R.J. (1989) *Method of catalyzing chemical reactions.* U.S. Patent 4,888,281.
- Massey, R.J. et al. (1999) *Methods for improved particle electrochemiluminescence assay.* U.S. Patent 5,935,779.
- Massey, R.J. et al. (1999) *Graphitic nanotubes in luminescence assays.* U.S. Patent 5,866,434.
- Leland, J.K. et al. (1999) *Methods and apparatus for improved luminescence assays.* U.S. Patent 5,962,218.
- Leland, J.K. et al. (1998) *Apparatus for improved luminescence assays.* U.S. Patent 5,779,976.

Mark W. Surber, Ph.D. will be the Senior Laboratory Scientist. Dr. Surber has substantial experience in molecular biology. He is currently working with IGEN's human antibody phage display library and with IGEN's electrochemiluminescence analytical instrumentation. Prior to joining IGEN, Dr. Surber received a Ph.D. degree in Microbiology from the University of Illinois in 1999. Before that, he recieved an M.S. degree from California State University, Long Beach, also in Microbiology. Prior to joining IGEN, his primary research interest was the genetic regulation and protein-lipid interactions of membrane-associated flavoproteins. In particular, he studied the molecular biology and enzymology of proline utilization in *Salmonella typhimurium.*

II. RESEARCH PLAN

A. SPECIFIC AIMS

1. Overview

The goal of the proposed project is to apply new and powerful research methodologies to discover therapeutic catalytic antibodies (Abzymes®[1]) to prevent and/or treat acute solid organ transplant rejection. These therapeutic abzymes will selectively inactivate CD3ε, a part of the T-lymphocyte receptor that is involved in the process of acute rejection. Success in the project will yield highly effective immunosuppressive agents and will also validate discovery tools that can be used to create catalytic antibodies to treat numerous other diseases, including cancer and autoimmune diseases.

Catalytic antibodies are structurally identical to antibodies, but functionally similar to enzymes. Each abzyme molecule can react with, and therefore inactivate, multiple disease-associated target molecules. Although abzymes have been shown to catalyze as many as 20 turnovers per second, a typical catalytic antibody (catAb)[2] may carry out seven turnovers per minute (Jacobs, 1991). If a therapeutic abzyme were to circulate in the bloodstream for 24 hours catalyzing seven turnovers per minute, it will have inactivated 10,000 target molecules (compared to one target molecule for a monoclonal antibody). Therefore, catAbs promise to offer substantial improvements over existing antibody therapies in efficacy and cost effectiveness.

Although considerable research has been carried out on potentially therapeutic catalytic antibodies (Smithrud and Benkovic, 1997), few advances in this field have been commercialized (Martin, 1996). The main obstacles to commercialization have been: (1) the focus on developing abzymes to catalyze kinetically challenging reactions, such as
* proteolysis, and (2) the labor intensive process of discovering and screening abzymes for activity. We plan to overcome these barriers by combining several technological innovations. First, rather than using hydrolysis as a target inactivation reaction, we will use kinetically facile chemical modification reactions. Second, we will use two new abzyme discovery methods, directed evolution and high throughput screening, to identify and screen candidate catAbs for efficacy. These two methods are rapid, versatile and laborsaving, and will be used in parallel.

Our plan is to discover abzyme(s) that inactivate the graft rejection-associated T-cell receptor protein CD3ε. We further plan to show that the discovered abzymes have desirable biological effects in appropriate cell and animal disease

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[1] Abzyme is a registered trademark of IGEN International, Inc.

[2] Abbreviations: catAb, catalytic antibody or abzyme; ECL, electrochemiluminescence; HTS, high throughput screening; IgG, immunoglobulin G; mAb, monoclonal antibody; $Ru(bpy)_3^{2+}$, ruthenium (II) tris(bipyridine); scFv, Single Chain Variable region Fragment of an antibody; TSA, transition state analog.

models. We eventually hope to test at least one abzyme in human clinical trials and to ultimately commercialize it as an immunosuppressive agent to prevent or to treat transplant rejection. All of the innovations described in this proposal have broad applicability and could be exploited to discover abzymes to treat a variety of diseases.

2. Technical Milestones

The usual time to develop a drug from the discovery phase to FDA approval is 12-15 years. The work described in this proposal constitutes the initial high-risk R&D phase of therapeutic abzyme development. Continued technology development and human clinical trials will be funded separately. The discovery and development of anti-CD3ε abzymes can be broken down into defined research stages, outlined below as our Phase I milestones.

Phase I Milestones: Anti-CD3ε Catalytic Antibody Discovery and Development

1) Human CD3ε cloning and expression
2) Measurement of CD3ε/antibiotic uncatalyzed rate
3) Development of an electrochemiluminescence-based (ECL) immunoassay for CD3ε
4) High throughput screening of a human antibody library to discover anti-CD3ε catAbs
5) Directed evolution of human antibody library to discover anti-CD3ε catAbs
6) Characterization of discovered anti-CD3ε catAbs
7) Re-engineering the structure of a selected anti-CD3ε catAb from an scFv to an IgG
8) Re-characterization of the anti-CD3ε catAb

Anticipated Phase II Milestones: Anti-CD3ε Catalytic Antibody Therapeutic Trials

8) Biological (cell and animal studies) to test efficacy of anti-CD3ε catAbs
9) Production and preclinical testing of pharmacokinetics and safety in animals.
10) Begin human clinical trials in transplant patients

B. SIGNIFICANCE

1. Technical Challenges and Proposed Solutions

Catalytic antibodies (abzymes) are antibodies whose combining sites have been engineered to have enzyme-like catalytic activity (Martin, 1996). Antibodies are more attractive than enzymes as starting points for designing novel biocatalysts. In contrast to enzyme active sites, nature has endowed antibodies with combining sites that have remarkable malleability. The immune system evolved the "immunoglobulin fold" to be readily transformable to accommodate virtually any molecule. In contrast, enzymes have active sites that evolved for a single substrate and chemical reaction.

Catalytic antibodies first appeared in the scientific literature in 1986 (Tramontano et al., 1986; Pollack et al., 1986). Since then, hundreds of journal articles on catAbs have been published that describe: different types of catAb-mediated reactions; high-resolution x-ray crystallographic structures of the proteins; detailed mechanistic studies; and mutagenesis (Smithrud and Benkovic, 1997; Martin, 1996). Despite all of this research, there are currently only two examples of commercial, or near commercial, functional catAbs. Aldrich Chemical Co. offers an aldolase catAb that is useful in catalyzing a number of industrially relevant reactions. Also, a catAb that hydrolytically inactivates cocaine is currently under development as a detoxifying agent by MedImmune, Inc. (Gaithersburg, MD) and Columbia University.

An often-cited antibody-catalyzed reaction with enormous commercial potential is specific peptide bond hydrolysis. Efficient proteolytic inactivation of disease-associated proteins or peptides would have therapeutic potential in virtually every disease state, including cancer, viral and bacterial infectious diseases, and heart disease. Unfortunately, despite significant effort in many laboratories, no efficient peptide bond-hydrolyzing abzymes have been developed. Why has this research yielded so few successes?

- First, peptide bonds are extremely stable. Estimates of the half-life for uncatalyzed hydrolysis of peptide bonds range from 7 to 243 years, depending on the amino acid sequence (Smith and Hansen, 1998; Angeles et al., 1993; Kahn and Still, 1988). Because of the slow uncatalyzed rates, commercially viable abzymes must provide tremendous rate accelerations (k_{cat}/k_{uncat}).

- Second, catalytic antibodies are produced by a labor-intensive process of designing and synthesizing haptens that are analogs of the transition state of the target reaction[3] (up to one year), immunizing mice and hybridoma formation (6 months), and antibody screening for catalysis (1-2 months). This painstaking process often results in no catalysts. Several potential reasons have been suggested for this failure. One is that transition state analogs (TSAs) do not sufficiently mimic the true transition state of the reaction. Another possibility is that the number of antibodies screened for catalysis is too small (generally 10-50 are screened) to discover rare, highly efficient abzymes (Smithrud and Benkovic, 1997).

We plan to overcome these shortcomings by using an alternative reaction scheme to peptide bond hydrolysis and by using two new and innovative methods for discovering and screening abzymes:

- *Abzyme-Catalyzed Chemical Modification* of target proteins. In these reactions, the abzyme catalyzes a bimolecular reaction that modifies a specific amino acid residue in a target protein. Chemical modification reactions are much more kinetically facile than peptide bond hydrolysis, yet both result in protein inactivation. In bimolecular reactions, if the antibody binds both substrates simultaneously in a productive orientation the reaction will be greatly accelerated by a proximity effect. This method does not require synthesizing a transition state analog of the reaction, but rather the generation of antibodies that bind to a compound that resembles the two substrates or the product. Although this strategy has met with some success (Hirschmann, 1997), it is not often used since most abzyme reactions of interest involve a single substrate. In our project, abzymes will be made to inactivate target proteins by acylation with β-lactam antibiotics.
- *Directed Evolution.* Directed evolution (Joyce, 1994; Arnold, 1998) can be defined as the man-made generation of a large number of mutants of a chosen gene, followed by the selection of mutant genes that express a protein with desired characteristics. Directed evolution differs from natural evolution in two key respects. Firstly, genetic variation is introduced by the experimenter rather than by nature (Black and Loeb, 1993). This allows a greater number and diversity of mutants to be created faster than would occur naturally. Secondly, whereas natural evolution occurs by the process of natural selection (i.e., survival of the fittest), in directed evolution novel proteins are obtained by either natural selection (e.g., using auxotrophic bacteria (Tang et al., 1991)), or by individual or bulk screening of the generated novel proteins (e.g., high throughput screening (Grayling, 1998) or phage display (Fujii et al., 1998)).

  We will use directed evolution as a natural selection method to obtain efficient abzymes. *E. coli* bacteria will produce and secrete single chain antibodies. Bacterial colonies that possess appropriate catalytic activity will survive under the experimental conditions, while those that do not have catalytic activity will die.
- *Electrochemiluminescence-Based High Throughput Screening.* High throughput screening (HTS) is a popular drug discovery method (Bolger, 1999). Generally, thousands of assays to detect candidate drugs can be performed per day. Screening usually involves multi-well microtiter plates (96, 384, or 1536-well plates) and robotics to transport and measure liquid samples. Some common analytical methods used in HTS are absorbance, fluorescence, radioactivity, luminescence, and electrochemiluminescence (ECL).

  IGEN's two pillars of intellectual property are ECL (Blackburn et al., 1991; Massey, 1992) and catalytic antibodies (Martin, 1996). We plan to use ECL as a tool in the discovery of catalytic antibodies. Using appropriate ECL-based immunoassays, we will screen thousands of antibodies for catalytic activity. Because we will screen many more antibodies for activity than has previously been practical, we hope to discover rare, highly efficient catalytic antibodies that would be missed by using conventional methods. To our knowledge, no catalytic antibodies have ever been discovered using HTS. ECL-based catAb discovery is now especially attractive with the launch of IGEN's new M-SERIES high throughput screening instrument, which is sold to pharmaceutical discovery laboratories.

2. Benefits of Abzymes over Monoclonal Antibodies

Therapeutic monoclonal antibodies (mAbs) have begun to have a dramatic impact on the practice of medicine. The fruit of extensive research and development, these drugs target specific disease-associated proteins and so provide therapeutic benefit while minimizing side effects. They represent 13% of drugs on the market and 24% of those in the development pipeline (Nelson, 1999; p.6). Worldwide revenues for mAb drugs were $1.4 billion in 1999 and are expected to grow to $8.1 billion by 2004 (Nelson, 1999; p. 17).

[3] The transition state is a fleeting (10^{-13} s) high-energy intermediate that appears during the course of the reaction. Enzyme theory states that enzymes are catalytic because their active sites are complementary to the transition state. Antibodies raised bind to a transition state analog should also be complementary to the actual transition state, and thus they should be catalytic.

Like mAbs, catalytic antibodies offer exquisite specificity to disease-associated proteins. However, unlike mAbs, each abzyme molecule can react with multiple target molecules, thereby permitting dramatic enhancements in therapeutic efficacy. Lower doses would be required, in principle, to achieve the same result. This would lead to a reduced incidence of harmful side effects, which are typically dose dependent. Additionally, since the catalytic antibodies will be fully humanized, immunogenic side effects should be virtually absent. Thus, the therapeutic index of abzymes is expected to be higher than for mAbs. These new therapeutic proteins should contribute greatly to improving the health of a large number of Americans and could displace mAbs as the state-of-the-art in immunotherapeutics.

In addition, to an improved therapeutic index, lower doses would result in lower treatment costs to the patient. In the technical proposal, we estimate the potential enhancement in efficacy of an abzyme drug, as compared to conventional antibody therapy, to be on the order of 10^4. This implies that the therapeutically effective dose of an abzyme will be about 0.01% that of a conventional antibody drug. (The most effective dose will depend, of course, on the pharmacokinetics of the catAb in the body.) If manufacturing costs for both types of compounds are comparable, the cost savings associated with catAb therapies will be commensurate with the dose size. Current costs for monoclonal antibody therapies are in the range of $10,000 to $15,000 per patient per year.

Another potentially significant benefit of abzymes over mAbs is the possibility of oral administration. Since each catAb molecule is capable of reacting with multiple target molecules, one could envision administering an oral dose of abzyme that is comparable to an intravenous dose of mAb, and still achieving a therapeutically effective drug level in the bloodstream despite degradation of a portion of the dose in the stomach. Of course, in this case, the benefits of lower doses and drug costs might not be achieved; but the possibility of avoiding patient inconvenience and anxiety related to intravenous administration might outweigh these other benefits.

3. Abzymes to Prevent and Treat Transplant Rejection

During the proposed SBIR project, IGEN will develop candidate therapeutic catalytic antibodies to prevent and/or treat solid organ transplant rejection. About 20,000 patients receive organ transplants in the United States each year. Rejection is the most common cause of transplant failure, occuring in greater than 80% of solid organ transplant recipients. In order to prevent or treat this potentially fatal immune system response, transplant patients must take immunosuppressive medications. Depending on the patient's condition, different therapies are mandated. Small molecule immunosuppressive drugs such as cyclosporine or tacrolimus, mycophenolate mofetil or azathioprine, and prednisone are taking prophylactically post-surgically to reduce the likelihood of rejection. In cases of acute rejection, where the patient's T-cell lymphocytes attack the graft, higher doses of immunosuppressants, corticosteroids or monoclonal antibody therapies are administered. Current mAbs for acute transplant rejection are: muromonab-CD3 (OKT3, Orthoclone OKT3) by Ortho/J&J which targets the CD3ε protein; basiliximab (Simulect) by Novartis; and daclizumab (Zenapax) by Roche. The latter two antibodies are anti-IL2 receptor agents and are also administered before and after surgery to reduce the likelihood of rejection in the first year. OKT3 is administered prophylactically only in cases where the likelihood of rejection is high (Wilde and Goa, 1996).

Small molecule drugs have the advantage of low cost and relative ease of administration, but they are unselective in their immunosuppressive effects and patients taking them can suffer from severe side effects and are at increased risk of developing infections and lymphomas. Monoclonal antibodies are much more specific in their mode of action, targeting proteins involved in the pathological process. However, these drugs also can have significant side effects. OKT3, in particular, elicits immunogenic side effects as a result of being a murine antibody. The Fc region of the antibody causes T-cell cross-linking, which can result in a first dose syndrome of T-cell activation and cytokine release that results in toxicity and sometimes death (Todd and Brogden, 1989; Glaser, 1999). Protein Design Labs has re-engineered the Fc portion of an anti-CD3ε antibody (HuM291, SMART anti-CD3) and has had success with this agent in early clinical trials (Klingbeil and Hsu, 1999).

The advantages of high specificity and reactivity (and therefore low cost), should allow abzymes to compete successfully against both small molecule drugs and therapeutic mAbs for preventing and treating transplant rejection. Catalytic antibodies will be at a competitive disadvantage relative to mAbs with respect to market timing, since mAb therapies are gaining market share and will have been around for more than a decade before the first FDA-approved therapeutic abzyme is approved. We believe, however, that the growing acceptance of antibody therapeutics in the medical and regulatory communities makes this an opportune time for IGEN to launch a program in catAb research and development. The current generation of mAb drugs is creating new markets for biological therapeutics that IGEN will be able to tap with its improved abzyme therapies. In essence, catalytic antibodies could be marketed as the next generation of immunotherapies.

Furthermore, as more clinical studies are carried out on both the efficacy and long term effects of mAb therapies, the therapeutic indications of these drugs will be further refined. IGEN will benefit from these studies, in that they will help us to anticipate potential pitfalls in the development and validation of our antibody drugs and allow us to design more effective clinical trials. New, more efficient manufacturing techniques for therapeutic mAb production will also be available to us as we explore our options for commercial production.

Finally, by focusing on a disease-associated target for which validated antibody therapies already exist, our R&D strategy minimizes the uncertainty associated with target selection. One of the most difficult aspects of drug development is choosing the appropriate disease-associated protein to target. In the case of autoimmune disorders and transplant rejection, for example, several cytokines are involved in the disease pathway. Identifying key target molecules depend on a detailed biochemical understanding as well as trial-and-error research. We will therefore be leveraging the research that served to validate the disease targets for current mAb therapies. By focusing on validated targets, we will also be in a stronger strategic position to collaborate with therapeutic antibody companies that already have approved mAb therapies on the market for these targets.

C. RELEVANT EXPERIENCE

1. Experience and Qualifications

Dr. Mark T. Martin, the principal investigator, has experience in all aspects of the proposed research. Dr. Martin has been with IGEN for eleven years and has carried out research in catalytic antibodies, phage display, and electrochemiluminescence. He has published numerous peer-reviewed journal articles and holds several patents in the fields of catalytic antibodies, phage display, and electrochemiluminescence.

Dr. Richard J. Massey, Senior Advisor on the project, is a founder of IGEN International, Inc., and is currently President and COO. He is an early inventor of catalytic antibodies and also holds numerous other patents in catalytic antibody and ECL technology.

We note that IGEN's CEO and Chairman, Sam Wohlstadter, is also CEO of three privately held biotechnology companies that are focusing on developing novel therapeutic compounds and viruses. Two of these companies, Pro-Neuron and Pro-Virus, have three therapeutics in Phase II trials and two in Phase I trials for cancer treatment, promoting bone marrow transplantation, and wound healing. Mr. Wohlstadter has many years of experience as an executive and a venture capitalist and will be invaluable in helping to establish relationships with strategic partners during the commercialization phase of the program.

2. IGEN Capabilities and Intellectual Property

In this section, we will describe the technical expertise of IGEN staff. We will also discuss IGEN's substantial intellectual property in the fields described.

Catalytic Antibodies and Affiliated Technologies

IGEN has a history in catalytic antibodies dating back to 1983 when patent applications disclosing catalytic antibodies were first filed by Gerald Schochetman and Richard Massey. The resulting patents constitute the first and broadest in the field. IGEN has a dominant patent position covering catalytic antibodies and their therapeutic and diagnostic uses (31 issued and 21 pending US patents, and 74 issued foreign patents), extensive relevant scientific know-how, and broad expertise in developing commercial analytical instrumentation and assays for drug discovery.

IGEN's scientists have technical expertise in all aspects of the proposed project, including making, testing, and characterizing catalytic antibodies. The staff includes highly talented organic chemists, protein chemists, molecular biologists, enzymologists, and microbiologists. In addition, IGEN has experts in the field of antibody phage display, high throughput screening, ECL, and directed evolution. Staff scientists have many scientific publications and patents in catalytic antibodies, ECL, phage display, and directed evolution.

IGEN's experience with phage antibody display spans almost 9 years, a time period that encompasses the full development cycle for this technology, since its first description in the scientific literature. IGEN was one of the first companies to form a corporate alliance with Cambridge Antibody Technology (CAT), a U.K. based company that originally developed the technology and that holds a number of key patents in the field. Through this collaboration, IGEN obtained exclusive rights to use the technology to develop catalytic antibodies. In addition, the research collaboration between IGEN and CAT resulted in the filing and issuance of two patents that encompass many basic aspects for the generation and selection of catalytic antibodies using

phage display. In addition to licenses from CAT, IGEN has also has a non-exclusive license for phage display technology from a privately held U.S. company, Dyax.

In 1994, IGEN established a research group within its R&D department to further develop phage antibody display technology primarily to support reagent and assay development for its core ECL business. An important highlight of this research effort was the construction and validation of a large scFv repertoire display library derived solely from non-immunized human donors. The size and complexity of this library allows one to isolate fully human, high-affinity scFv binding reagents to virtually any antigen. In addition, the in-vitro nature of the selection process eliminates the need for immunization and shortens the time for antibody development from months to weeks. In developing antibodies for therapeutic use in humans, it is especially important that the immunoglobulin domains are derived from human rather then mouse sources. Mouse derived therapeutic antibodies are generally recognized as foreign proteins when administered to humans, particularly if given in multiple doses. The resulting human-anti-mouse-antibody or HAMA response can severely compromise the therapeutic effect of the antibody. The use of a fully human phage antibody library for developing therapeutic abzymes obviates the need for the often expensive and lengthy process of "humanization" that would be required if using a mouse derived monoclonal antibody.

Electrochemiluminescence and High Throughput Screening

IGEN has an extremely strong patent position in the field of electrochemiluminescence. The company has 41 issued US patents and numerous foreign patents relating to ECL, including the broadest pioneering patents. With the exception of IGEN and its partners, no companies are involved in developing this technology.

ECL is an exquisitely sensitive and robust analytical method capable of precisely quantitating the specific binding of any two molecules. In this method, light is generated as a result of labelled molecules undergoing electrochemical reactions at an electode surface. IGEN has exploited ECL to carry out immunoassays, DNA probe assays, and clinical chemistry assays. IGEN manufactures and markets two ECL analyzer product lines, the ORIGEN Detection System and the recently launched M-SERIES High Throughput Screening system for use by biopharmaceutical companies in the drug discovery process. In addition, the company manufactures and markets GMP certified reagents for *in vitro* diagnostic testing and monoclonal antibodies for research use, and offers assay development services to pharmaceutical clients.

The M-SERIES system is based upon our second generation ORIGEN-based system, the ECL Module (ECLM). The ECLM combines small size and relatively low cost, with speed, accuracy and sensitivity equal to the first generation of ORIGEN-based products. The M-SERIES instrument, which comprises several ECL modules, is network- and robot-compatible, draws and reads liquid samples for 96- and 384-well microtiter plates. The rate of throughput is 1 plate (96 readings) per 10 minutes. Hundreds of M-SERIES-compatible ECL assays have been developed.

D. EXPERIMENTAL DESIGN AND METHODS (Most of Section D is proprietary)

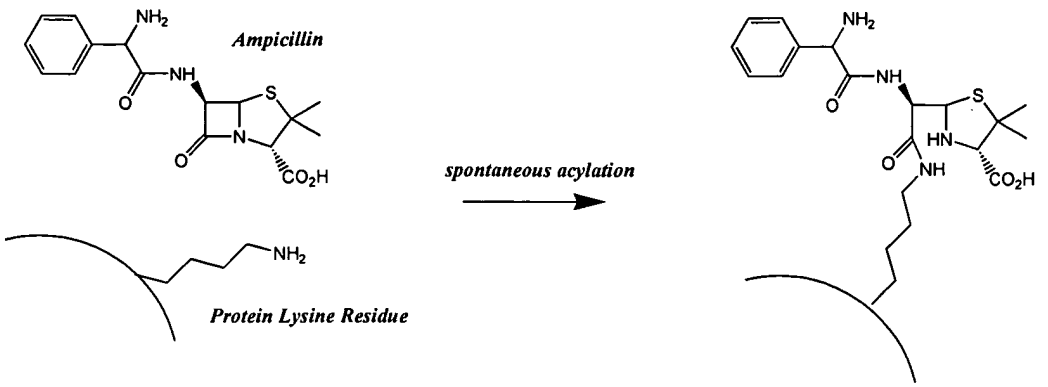
In this section, we give a detailed description of the research plan. First, we describe the three innovations we will use:
* (1) protein inactivation by abzyme-catalyzed chemical modification, (2) abzyme discovery using directed evolution, and (3) abzyme discovery using ECL-based HTS. Next, we give detailed descriptions of the discovery of therapeutic abzymes directed against the protein target CD3ε.

1. Techniques

Protein Target Inactivation by Chemical Modification

Concept and Advantages: As an alternative to proteolysis, proteins and peptides can be inactivated by chemical modification
* reactions – reactions that are much more facile than peptide bond hydrolysis.

There are well-known examples of uncatalyzed chemical modification reactions in the bloodstream. For example, the aldehyde group on circulating glucose spontaneously reacts with protein lysine residues to form covalent "advanced glycation
* endproducts" (AGE). The resulting glycated proteins cause diabetic complications. Another example is the acylation of protein lysine residues by β-lactam antibiotics (Simon et al., 1993) (see scheme below). Individuals who have penicillin allergies are actually not allergic to the penicillins themselves, but rather to penicillin-protein (usually albumin) conjugates (Magi et al., 1995). If abzymes could be used to direct chemical modification reactions to inactivate specific target proteins, they could be very effective therapeutic agents.



Feasibility: We carried out feasibility studies to determine whether antibody-catalyzed chemical modification is a viable approach to inactivating disease-associated proteins. First, we examined the reaction of reducing sugars with the disease-associated protein, tumor necrosis factor alpha (TNFα). TNFα is a plausible therapeutic target. In this reaction, the reducing sugar aldehyde covalently reacts with protein lysine sidechains. We detected chemical modification by using an ECL-based immunoassay that measures the binding of TNFα to its receptor (an assay of biological function). The results (below) show that sugars gradually inactivate TNFα receptor binding over a 2-week incubation period. In a separate experiment, we showed that the non-reducing sugar, sucrose, did not inactivate TNFα (data not shown). Thus, chemical modification is a viable approach to inactivating proteins.

Loss of TNFα Receptor Binding Following Incubation with Various Sugars

Incubation Time	Control (buffer)	+ Glucose	+ Galactose	+ Fructose
None	1.00 (arbitrary)	1.04	1.00	1.02
7 days	1.00 (arbitrary)	0.82	0.89	0.79
14 days	1.00 (arbitrary)	0.82	0.82	0.67

We also looked at the uncatalyzed reaction between a β-lactam (ampicillin) and two proteins. Bovine serum albumin (BSA) and ampicillin were reacted overnight. Subsequent chemical analysis showed that ampicillin groups were covalently attached to BSA. We also reacted ampicillin overnight with TNFα. We found that ampicillin caused a 16% loss of TNFα biological activity. This reaction is thus much more facile than the reaction of TNFα with reducing sugars. This work suggests that β-lactam antibiotics could be effective modification labels in abzyme-catalyzed inactivation of therapeutically relevant target proteins.

Directed Evolution to Create Catalytic Antibodies

Concept and Advantages: β-Lactam antibiotics (including the penicillins and cephalosporins) are toxic to bacteria when the four-membered heterocyclic β-lactam ring structure is intact, but are completely non-toxic after the ring is opened by hydrolysis or acylation. We plan to use directed evolution to discover abzymes that covalently inactivate target proteins by acylation with β-lactam antibiotics. Antibodies in an antibody library will be individually expressed in and secreted by *E. coli*. The target protein will either be added to the growth medium or co-expressed with the antibody. A toxic level of β-lactam antibiotic will be added to the *E. coli* colonies. *Any organism that secretes an abzyme that can catalyze the acylation of a target protein with antibiotic will survive because the process of acylation (ring opening) inactivates the antibiotic.*

Integration with Phage Display: Phage display (Wilson and Finlay, 1998) is a technology in which large collections of filamentous bacteriophage particles (often exceeding 10^{10} unique particles) are used as tools to discover unique peptides or proteins. All of the phage in a library are physically identical except that each particle displays 1-5 copies of a unique protein or peptide on its surface. By applying a specific selection method (binding or catalysis) to the bulk phage library, phages displaying proteins with chosen properties can be isolated. The beauty of the technology is that the isolated phage particles physically contain the gene that encodes the displayed protein. Hence, the peptide/protein can be easily scaled up, purified, and characterized.

Many types of peptides and proteins have been used in phage libraries, including small peptides (Chiniros-Rojas et al., 1998), enzymes (Demartis et al., 1999), and antibodies (Winter et al., 1994). Phage libraries have been used to isolate candidate therapeutic antibodies (Huls et al., 1999; Mao et al., 1999) and catalytic antibodies (Arkin and Wells, 1998; Fujii et al., 1998).

The high molecular weight disulfide-linked tetrameric structure of natural IgG molecules is difficult to express in E.coli, therefore the preferred form for antibody phage display is the single chain Fv or scFv (Huston et al., 1993). The 25 kDa scFv molecules consist of only the variable heavy and light chain regions of antibodies, connected by a short peptide linker, which fold to form a functional antibody binding site. If desired, scFv molecules can be easily re-engineered to Fab, full-sized IgG, or other molecular forms.

It is a tremendous advantage for us to have a library of antibody genes for functional expression and display on phage particles. Since the process to assemble and display the immune repertoire on the phage is performed *ex vivo* it is likely that the potential numbers of antibody fragments with catalytic activity is much larger then would be found *in vivo*. This is theoretically possible because the pairing of the variable heavy and light chain domains that comprise the binding pocket is: 1) completely random and combinatorial, and 2) not restricted in any way by normal immunological regulation.

IGEN has a diverse scFv phage library consisting of approximately 10^{12} unique antibodies. This antibody library will be the source of our abzymes. Prior to performing the directed evolution experiments, we will selectively reduce the size of the phage antibody library from ~10^{12} to ~10^4 based on enrichment binding to the reaction product. There are two reasons for pre-selecting antibodies on the basis of product binding. First, due to the nature of phage display technology, only a portion of the phage in the library display a functional scFv. Pre-selection will enrich those that display an scFv. The second reason is that directed evolution generally is aided by using a subset of potential compounds that is has a higher likelihood of containing the desired catalyst. It is easier to go from "somewhere" to "somewhere else" in the vast sequence space than to create function *de novo*. Thus, by pre-selecting a subset of 10^4 antibodies that bind, at least weakly, to the reaction product (antibiotic-target adduct), the chances of finding a catalyst are enhanced.

To pre-select ~10^4 antibodies that have some affinity for the product, we will first use the uncatalyzed reaction to chemically prepare the β-lactam-target protein conjugate (see above). The target antigen will be generated in a purified or partially purified form (i.e. 50 to 90% homogeneity). We will then "pan" the human antibody phage library against the conjugate. In panning, phage antibodies are incubated in a plastic tube containing surface-coated antigen. A wash step is employed to strip off non-specifically bound phage, after which bound phage are removed by elution with a high pH buffer. Typically, multiple rounds of panning are carried out. In our work, we need to retain as much diversity as possible. Thus, we will limit the selection process to a single round of panning and will minimize the number of wash steps of the tube after antigen selection and prior to elution. The expected number of clones eluted from the tube after a single panning is on the order of 1-5 x 10^4, which is amenable to our plans.

After phages are panned to reduce the library size, we will infect *E. coli* with the resulting phage and use this sub-library in directed evolution work. We note that the same 10^4 antibodies will be used in the HTS experiments described below.

Although we have demonstrated the utility of our phage antibody library for generating specific binding reagents, this work represents our first attempt at isolating defined catalysts.

Feasibility: Directed evolution of catalytic antibodies has been carried out previously using both bacterial (Smiley and Benkovic, 1994) and yeast (Tang et al., 1991) based systems. In both examples the abzyme conferred a survival advantage through the generation of an essential element for growth under selective conditions. No one has previously obtained (or sought to obtain) designer biocatalysts that conjugate antibiotics to target proteins, by directed evolution or otherwise. This method of directed evolution is untested and represents significant risk.

β-Lactam antibiotics have been used previously as the selection pressure against *E. coli* in directed evolution studies (Black and Loeb, 1993; Zaccolo and Gherardi, 1999; Crameri et al., 1998; Osuna, 1994). In those cases, the bacterial enzyme β-lactamase was the protein subjected to directed evolution. β-Lactamase hydrolytically destroys β-lactam antibiotics such as the penicillins and is usually responsible for bacterial resistance to antibiotics. This enzyme is particularly amenable to directed evolution since mutant enzymes with improved catalytic activity will give the host organism greater antibiotic resistance. Thus, those bacteria with efficient mutant enzymes will survive an antibiotic challenge. As there are no practical applications of β-lactamase, the directed evolution studies were carried out as a convenient way to test academically interesting principles. These studies show the feasibility of using β-lactams in directed evolution work.

Although we demonstrated that the reaction of ampicillin with proteins was facile and gave the desired result (biological inactivation), there are two problems with using penicillins such as ampicillin as the chemically modifying reagent: 1) the scFv expression plasmid in the phage antibody library encodes a penicillin-hydrolyzing β-lactamase, and 2) penicillin allergies will ultimately limit the utility of abzyme therapies based on penicillin substrates.

We sought to identify β-lactam antibiotics that were not allergenic (cephalosporins rather than penicillins) *and* not recognized and hydrolyzed by *E. coli* β-lactamase. We identified four candidate cephalosporins for testing. All are FDA-approved

antibiotics and fairly inexpensive from a common commercial source (Sigma Chem. Co.). They also do not have the same potential of being allergenic as do the penicillins (Anne and Reisman, 1995). The incidence of allergic reactions to cephalosporins is very low and any reactions that may occur are likely to be mild (e.g., rash, urticaria).

First, we tested the ability of these antibiotics ability to kill the relevant β-lactamase-producing *E. coli* (TG1 phage antibody *E. coli*). The results (table below) show that ampicillin, Cefaclor, and Cephalothin have little or no antibiotic effect at the indicated concentrations. This is presumably due to β-lactamase-catalyzed inactivation. However, both Cefoxitin and Cefotaxime showed excellent antibiotic effects. Presumably, neither is inactivated by *E. coli* β-lactamase.

Protocol:

1. Prepared 2XYT + 2% Glucose agar medium and added antibiotic solution to a final concentration of either 10 or 100 μg/mL. Poured plates and let solidify at room temp.
2. Diluted *E. coli* TG1 phage antibody library stock culture 1:75 in LB medium to an approximate $OD_{600} = 3.0$ or about 1.5×10^9 bacteria per mL.
3. Plated 100 μL of diluted bacterial stock on each plate and incubated at 30° C for about 65 hr.

Results:

Growth on each plate was compared visually to the control plate containing ampicillin. A score of 5+ (+++++) indicates that growth was comparable to that of the ampicillin plate.

Antibiotic	10 μg/mL	100 μg/mL
Ampicillin	+++++	+++++
Cefaclor	+++++	+++
Cephalothin	+++++	+++++
Cefoxitin	+/- (30 colonies)	-
Cefotaxime	-	-

A second experiment was performed in which *E. coli* was titrated with low levels of Cefotaxime. The data (below) show that the toxic level of Cefotaxime is between 0.12 and 0.37 μg/mL.

Cefotaxime Concentration (μg/mL)	# of *E. coli* colonies
0.00	Lawn
0.04	225
0.12	5
0.37	0
1.11	0

Thus, the concentrations of Cefoxitin and Cefotaxime that are toxic to β–lactamase-expressing *E. coli* are approximately 10 μg/mL (22 μM) and 0.12 μg/mL (0.25 μM), respectively. These low figures indicate that these antibiotics are unrecognized by β-lactamase. Concentrations of these antibiotics that would be appropriate in directed evolution studies would be slightly (about 2x) higher than these concentrations in order to ensure that no *E. coli* would survive without evolved mechanisms. Thus, we will challenge *E. coli* with 44 μM Cefoxitin and 0.50 μM Cefotaxime.

In our experience, *E. coli* in our system expresses antibody at approximately 90 nM. Thus, for a catalyst to hydrolyze one-half of the antibiotic challenge (which would bring it to the brink of survival), it would have to catalyze 244 turnovers (22 μM/0.09 μM) with Cefoxitin or 14 turnovers (0.25 μM/0.09 μM) with Cefotaxime. A requirement for multiple turnovers is critical to ensure that *E.coli* cannot survive by simple binding of the antibody to the antibiotic, but that catalysis is required to allow growth.

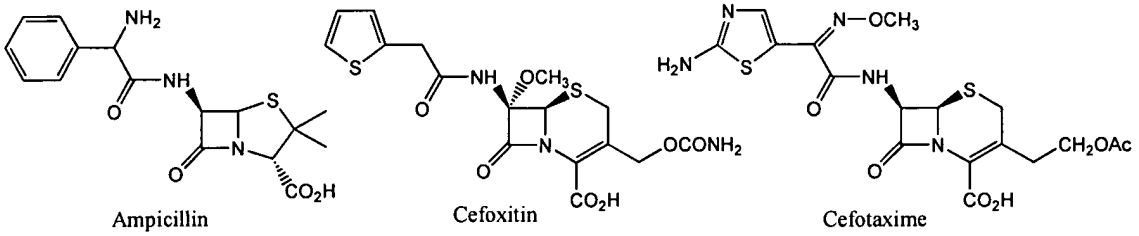
Before choosing either Cefoxitin or Cefotaxime as the labeling reactant, we verified that their uncatalyzed reactivity rates were similar to that of ampicillin (i.e. facile). We performed model reactions comparing the (NaOH) hydrolysis rates of Cefoxitin, Cefotaxime, and ampicillin. Hydrolysis and acylation are mechanistically identical. Their pseudo-first order hydrolysis rates (1.0 mM antibiotic, 10.0 mM NaOH, 30.0° C) are virtually the same:

Ampicillin	5.64×10^{-3} min^{-1}
Cefoxitin	4.38×10^{-3} min^{-1}
Cefotaxime	4.10×10^{-3} min^{-1}.

Both Cefoxitin and Cefotaxime are excellent compounds for our work:

- They are facile reactants.
- They are FDA-approved generic drugs; Cefoxitin (Mefoxitin) went off-patent in 1999 and Cefotaxime (Claforan) went off-patent in 1998.
- Because they are no longer covered by patents, they will be inexpensive.
- They are much milder allergens than penicillin β-lactams (Anne and Reisman, 1995).
- The concentrations at which they are toxic to *E. coli* make them ideal for the directed evolution plan described below.

We plan to pursue work with both Cefotaxime and Cefoxitin. Chemical structures of these two compounds, along with the penicillin ampicillin, are shown below.



Ampicillin Cefoxitin Cefotaxime

HTS to Create Catalytic Antibodies

Concept and Advantages: In this approach, IGEN will use phage display technology to screen a vast array (~10^{12}) of candidate compounds for binding activity. A subset of ~10^4 potentially active antibodies will be identified. The reason for using phage display to reduce the number of antibodies to be screened is mainly technical. It would be virtually impossible to screen a much larger number. The phage library will be panned using the reaction product (β-lactam-target protein conjugate), which will be prepared by the uncatalyzed reaction. Because panning is done with low stringency, a fairly large number (10^4) of binding antibodies are obtained. The antibodies are then grown in *E. coli.*

Secreted antibodies will be assayed for activity using electrochemiluminescence-based HTS (Blackburn et al., 1990). ECL-based HTS enables one to perform highly sensitive assays in a massively parallel fashion. The combination of phage display with ECL-based HTS will be unprecedented and may enable the discovery of rare, remarkably efficient abzymes. Although HTS of antibodies for catalysis is inherently risky because it has not previously been performed, it is relatively easy to format assays for ECL detection and IGEN has significant experience in developing ECL-based assays.

Integration with Phage Display: As stated above, we will subject ~10^4 antibodies to HTS. Notably, the same 10^4 antibodies will be used in directed evolution *and* HTS experiments. (See above for details.)

Feasibility: Can HTS can be used to identify abzymes effectively? The total number of antibodies we propose to screen is on the order of 10,000. The total number of assay wells required for the screen would be about 20,000, assuming each antibody test would be performed in duplicate. There will also be a minimal number of control wells (i.e., no added antibody). We expect to be able to complete the screen in approximately 10 working days assuming a conservative throughput of 2,000 wells/day. Such assay throughput is commonplace, as are most of the described assays. Several of the required ECL assay formats have been developed previously, and we expect the development time for additional formats will be relatively short due to the nature of ECL technology and IGEN's extensive assay development expertise. In addition to the ease of assay formatting, one of the major advantages of ECL compared to other technologies is the highly precise and reproducible nature of the assay results. The coefficient of variation (% CV) in signal for a typical ECL assay is < 10% and can be as low as 2% in a highly optimized assay. This assay precision should allow us to accurately quantitate relatively minor changes in ECL signal enabling the identification of even weakly catalytic antibodies.

2. The Target Molecule – CD3ε

We have carefully chosen the epsilon subunit of CD3 as an attractive protein target. Based on journal literature, functional inactivation will have a significant therapeutic effect. CD3ε was chosen following an exhaustive review of over 100 potential protein and peptide targets. CD3ε has the favorable criteria of:

- known 3-dimensional structure
- surface lysine residues believed to be critical for biological function
- numerous solid publications indicating that inactivation of the target will result in some therapeutic effect in an important disease state
- non-catAb therapies exist that show a beneficial effect that could improved by using a catalytic version. Existing non-catAb therapies are FDA-approved. Others are well along in published laboratory research and clinical trials.
- published cloning procedure and nucleotide sequence.

Furthermore, there have been problems with existing anti-CD3 immunotherapies because the Fc region causes cell-cell cross-linking (see below), which result in serious side effects. The use of catalytic antibodies will obviate this problem since the abzymes will only transiently bind to T-cell surfaces during catalysis. Typical durations of binding during the act of catalysis range from less than one second to a few seconds – less time than would be required for crosslinking to occur.

Physiological Role of CD3ε and Therapeutic Implications Antigen recognition by T-cells involves a complex between heterodimeric T-cell receptor (TCR) and the CD3 complex. CD3 consists of at least three different proteins, γ, δ, and ε. CD3ε is present on all T-cells and is absolutely required for T-cell activation (Elgart, 1996; Imboden, 1997).

Many antibodies that bind to CD3ε disrupt T-cell function, resulting in an immunosuppressive effect (Pescovitz, 1999; Bostrom and Ringden, 1995; Halloran and Prommool, 1998; Smith and Bluestone, 1997; Alegre et al., 1997). CD3ε is by far the most antigenic CD3 subunit, as most anti-CD3 antibodies bind to CD3ε (Tunnacliffe et al., 1989; Transy et al., 1989; Portoles et al, 1999). Because of their immunosuppressive activity, anti-CD3ε antibodies are effective in prevention and treatment of rejection of transplanted organs and bone marrow. Most anti-T-cell antibody treatments can deplete greater than 99% of circulating T-cells (Bostrom and Ringden, 1995). Anti-CD3ε antibodies may also be useful in the treatment of T-cell tumors (Ma et al., 1997).

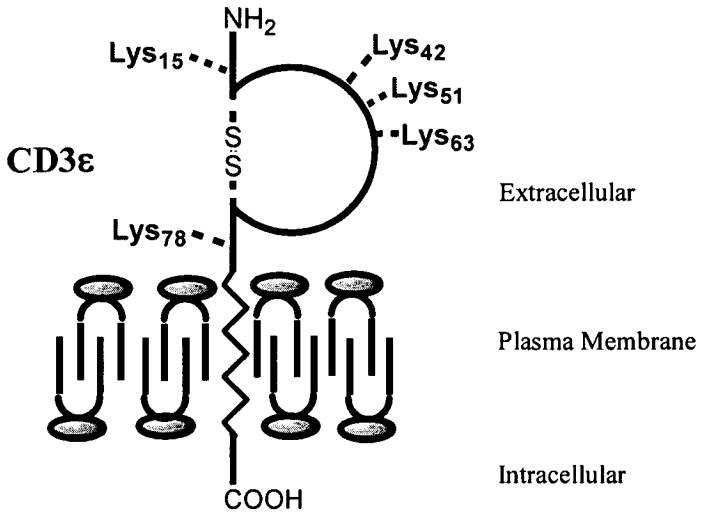
The first FDA-approved therapeutic monoclonal antibody was an anti-CD3ε antibody called OKT3 (Burk and Matuszewski, 1997; Pescovitz, 1999; Halloran and Prommool, 1998; Smith and Bluestone, 1997). OKT3, approved in 1980, is far from a perfect therapeutic agent because of severe side effects. Many of the adverse side effects arise as a consequence of OKT3 being a mouse antibody. In addition, because OKT3 has a Fc region, problems can occur due to cell-cell crosslinking. Ortho/J&J sells OKT3 (muromonab-CD3, Orthoclone OKT3) for use in transplantation.

Recently, improved anti-CD3ε antibodies have been reported that were prepared by humanizing mouse antibodies and inactivating the Fc region (Klingbeil and Hsu, 1999; Pescovitz, 1999; Smith and Bluestone, 1997). Protein Design Labs, Inc. has an engineered anti-CD3ε antibody that has done well in clinical trials (HuM291, SMART anti-CD3) (Klingbeil and Hsu, 1999). Most antibodies directed against CD3ε are naked antibodies, which act by blockading the biological activity of CD3. An alternative approach uses an anti-CD3 antibody to direct diphtheria toxin to T-cells (Ma et al., 1997). In addition, it should be noted that a number of other immunosuppressive antibodies (and other biologicals) are under development. Some would be redundant with anti-CD3 therapy (Halloran and Prommool, 1998; Alegre et al., 1997). For example, anti-IL2 receptor treatments are approved for transplantation – daclizumab (Zenapax, Roche) and basiliximab (Simulect, Novartis).

Structural Understanding of CD3ε: (Elgart, 1996; Imboden, 1997):

As mentioned above, CD3 consists of at least three different protein subunits, called γ, δ, and ε. The epsilon subunit is a 20-kDa non-glycosylated transmembrane protein. It consists of an amino-terminal 104 amino acid extracellular segment, a 26 amino acid hydrophobic transmembrane segment, and a 79 amino acid intracellular carboxyl terminus (see following figure; Elgart, 1996; Gold et al., 1996; Borroto et al., 1998).

CD3ε has a number of physical features that make it attractive as a target. It has been cloned, it is small (104 amino acid extracellular segment), and it is not glycosylated or otherwise post-translationally modified. Its folded structure is well understood. Five of the 104 extracellular amino acids are lysine residues – targets for catalyzed chemical modification (Elgert, 1996; Gold et al., 1986; Borroto et al., 1998). Anti-CD3ε antibodies are well-established therapeutic agents that have been in clinical use for 20 years.

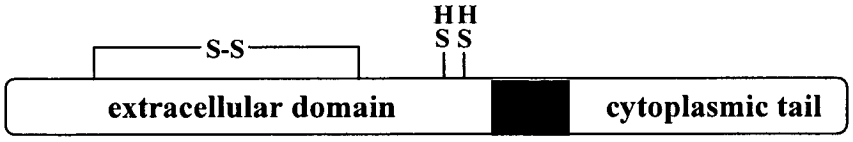


3. Experimental Details

Cloning and expression of human CD3ε

* For this part of the project, the extracellular 104 amino acid segment of CD3ε will be cloned, expressed, and purified as described. The cloned protein segment will be used as an abzyme substrate in HTS and in directed evolution.

A clone, which contains the coding sequence for the T-cell surface protein, CD3ε is available from ATCC (Manassas,VA). Once the clone is obtained the complete insert sequence will be determined by DNA sequencing and * compared to the previously published sequence of CD3ε cDNA (Gold et al., 1986). The protein consists of a total of 185 amino acids, with a structure shown in the diagram below (Huppa and Ploegh, 1997).



For bacterial expression of CD3ε, the DNA sequence corresponding to the extracellular domain will be amplified by PCR using specific primers. During this process, specific cloning sites will be added to the 5' and 3' ends of the amplified product, to allow subsequent cloning into an *E.coli* protein expression vector. A number of such vectors are available commercially, for example pET, which can be used to achieve high-level, secreted expression of the cloned CD3ε * protein. Secreted expression of the protein is an important aspect of the directed evolution approach for isolating catalytic antibodies that specifically modify CD3ε. In addition, the secreted CD3ε protein will be purified to homogeneity from *E. coli* cell paste for use in preparing the β-lactam-CD3ε protein conjugate. This conjugate is required for panning the phage antibody display library prior to isolating specific antibody catalysts using either HTS or directed evolution. Purification will be facilitated by expressing the CD3ε with a HIS_6 tail, a common component of most *E. coli* expression vectors, which allows for protein isolation in a single step using immobilized metal affinity chromatography or IMAC.

Two (reduced) cysteine residues (97 and 100) are in the extracellular domain near the transmembrane segment, which begins with valine 105 (see diagram above). Because thiols could cause technical problems due to oligomerization or * misfolding, we plan to remove these residues from the expressed protein. We will either use an appropriate restriction enzyme to eliminate the cysteines by gene truncation or perform site-directed mutagenesis to change them to alanine residues. For reference, the denoted extracellular loop is formed by a disulfide bridge involving cysteines 27 and 76. The exposed targeted lysine residues are numbers 15, 42, 51, 63, and 78. (Gold et al., 1986; Borroto et al., 1998)

Immunoassay Development

A number of anti-CD3ε monoclonal antibodies are commercially available that could be used in immunoassays to detect the chemical modification of CD3ε. The antibodies would be labeled with an activated NHS ester derivative of the ECL compound $Ru(bpy)_3^{2+}$ (IGEN Intl., Inc.) using an established protocol. It would be essential to choose an anti-CD3ε antibody that binds to native CD3ε but not to the β-lactam-CD3ε conjugate. It is also important that the antibody has been shown in the literature to disrupt T-cell function as a result of CD3ε binding (i.e., both the antibiotic and the antibody localize to a shared physiologically essential epitope). A number of apparently suitable antibodies are commercially available.

Assay development would first involve preparation of the antibiotic-CD3ε complex. As described, the spontaneous reaction can be carried out between the antibiotic(s) and cloned and expressed CD3ε for 2-3 days, resulting in modified protein. Mild neutral, aqueous conditions can be used. The conjugate can easily be purified by dialysis and column chromatography.

Labeled anti-CD3ε antibodies will then be screened for binding to the antibiotic-CD3ε conjugate. The conjugate will be immobilized according to standard ECL methods and antibody binding will be detected by light emission. Antibodies that bind to recombinant CD3ε but not to the antibiotic-CD3ε conjugate will be usable in immunoassays of catalytic activity.

Some sources of apparently appropriate anti-human CD3ε monoclonal antibodies are;
R&D Systems, Inc., Minneapolis, MN (Catalog # MAB100)
ATTC, Manassas, VA (antibody OKT3 (Cat. # CRL-8001) and antibody BC3 (Cat. # HB-10166))
BD Pharmingen, San Diego, CA (clones 1D4.1, 8D3.1, SP34)
Caltag Laboratories, Burlingame, CA (clone MEM57)
Accurate Chemical & Scientific Co., Westbury, NY (clone MEM57)
Research Diagnostics, Inc., Flanders, NJ (clone CLBT3-4E)

Phage Display

IGEN's human phage antibody repertoire display library will be panned against the antibiotic-CD3ε conjugate. The resulting subset of ~10^4 antibodies will be subjected to HTS and directed evolution.

The antibiotic-target protein conjugate for panning will be prepared by prolonged incubation of the two reaction components, CD3ε and antibiotic. The rate and yield of the uncatalyzed reaction will be optimized by varying the reaction conditions (time, temperature, pH, etc.). The rate of the reaction will be monitored by following the loss of CD3ε antibody binding. We will use the immunoassay described above to monitor the reaction progress. Appropriate controls will be used to ensure that the loss of CD3ε is not due to an artifact such as proteolysis or denaturation.

Once we have adequately formed antibiotic-CD3ε conjugate, the reaction mixture will be dialyzed to remove unreacted antibiotic and exchange buffer. The conjugate will be adsorbed onto a plastic tube. Next, the entire human scFv phage library (~10^{12} antibodies) will be added to the tube for panning. We will investigate various wash conditions to determine a suitable amount of washing to adequately reduce background phage binding without compromising the diversity of specifically selected phage. Bound phage will be eluted using pH shock and the resulting eluate infected into *E.coli* and plated on selective media to obtain isolated colonies.

HTS: Approximately 10,000 antibodies will be screened for catalytic activity during a two-week period. Phage antibodies will be expressed in *E. coli*, and the supernatants will be screened by immunoassay in an IGEN M-SERIES ECL instrument. Preparation for screening will take up most of the allotted time, and actual HTS for each target/antibiotic combination will only take 10 days. During those periods, 1000 antibodies will be screened for activity per day, in duplicate (about twenty 96-well plates per day). The M-SERIES instrument is capable of running five plates per hour. The CD3ε protein will be obtained as described in the phage display section above.

Directed Evolution: In directed evolution experiments, *E. coli* colonies representing the panned scFv library will be challenged with toxic doses of either Cefoxitin or Cefotaxime. If *E. coli* secretes an scFv that can catalyze the conjugation of the antibiotic to secreted CD3ε, then the antibiotic will be inactivated and that colony will survive. Colonies that do not secrete abzymes will not inactivate the antibiotic and will not be selected. The concentrations of antibiotic used will be twice the IC_{50}, or 44 μM Cefoxitin and 0.50 μM Cefotaxime (see above).

The simplest way to include the target protein and the antibiotic is to add them to the agar media used for the selection. This is reasonable for the antibiotic, but the amount of target protein required for selection on Cefoxitin would be on the order of

44 μM, which in the case of CD3ε would require as much as 500 milligrams to perform the selection using 1000 mL of media. The cost to perform the selection in this manner is prohibitive. Selection on Cefotaxime would require 10-fold less protein, which would be more cost feasible, provided we could produce the target protein at IGEN or by contract as opposed to directly purchasing from a vendor.

A second, more attractive approach is to co-express the target protein in the same bacterial cell as the scFv. The addition of a signal sequence will direct the expression of the protein to the bacterial periplasm, the same location as the expressed scFv protein. The two major advantages of this approach would be: 1) lower cost compared to adding the target protein to the media 2) a more favorable environment for catalysis as a consequence of concentrating the three components of the reaction in the bacterial periplasmic space.

Co-expression of the scFv and target protein in the same bacterial cell will be achieved by:

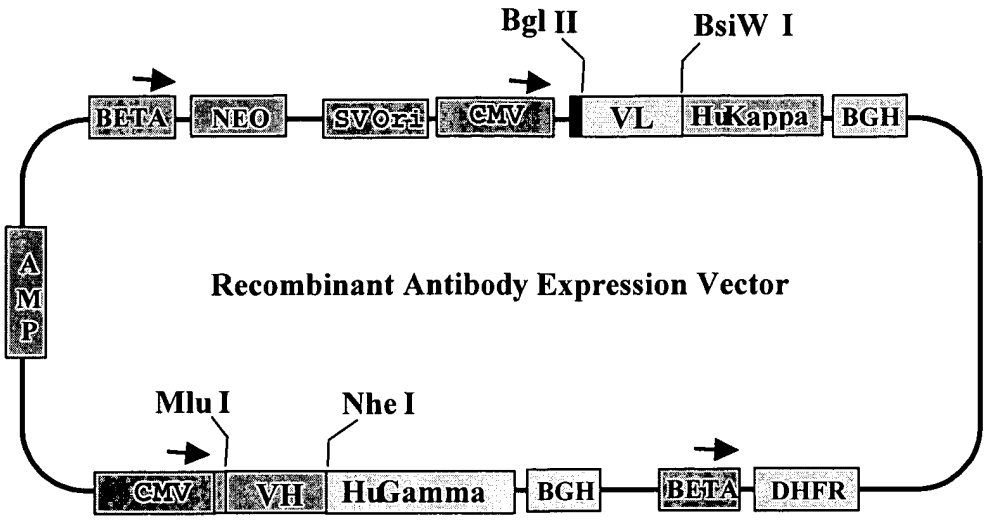
1. Following selection, eluted phage (about 10^4 pfu) will be infected into an *E. coli* strain that harbors a plasmid that expresses and secretes CD3ε.
2. The infected cells will be pelleted by centrifugation, resuspended in a suitable volume of media and plated on agar medium containing the appropriate concentration of Cefoxitin or Cefotaxime.
3. After a suitable incubation, any colonies that appear can be isolated, regrown and stored in glycerol at - 80° C. Expressed scFv from each colony will then be purified on a large-scale and tested for catalytic activity.

Characterization of Discovered Abzymes: Abzymes (scFv) discovered by either HTS or directed evolution will be produced in *E. coli*, then purified by standard methods. Catalytic activity will be verified on pure antibody. Characteristic kinetic parameters (k_{cat}, K_m and k_{cat}/K_m) will be determined. Finally, screening and characterization of any inhibitors, including substrate and product inhibition will be carried out. The most attractive abzyme in terms of efficiency and stability will advance to the next phase of the project, molecular engineering.

Catalytic activity of discovered abzymes will be determined by two methods. One method we will use is an indirect method. Here, abzyme-catalyzed conjugation reactions will be quenched at suitable times using 10 mM NaOH. Sodium hydroxide will terminate the catalyzed reaction, and hydrolyze intact (unreacted) antibiotic. Upon hydrolysis, the UV/Vis absorbance of free antibiotic will change substantially ($\Delta\varepsilon$ for Cefoxitin is 800 $M^{-1}cm^{-1}$ (290 nm) and 544 $M^{-1}cm^{-1}$ (330 nm) for Cefotaxime), allowing residual (unreacted) antibiotic to be measured. The second method is ECL-based immunoassay of the intact target molecule. This method directly indicates target inactivation, but is somewhat more time-consuming than the indirect method. Both methods will be used in a complementary manner.

Re-engineering Abzymes from scFv fragments to Whole IgG: Although the scFv antibodies are ideal for cloning, expression, and phage display, their small size relative to whole IgG may diminish their therapeutic efficacy due to its short serum half-life. Therefore, conversion of the scFv abzymes to an IgG will be essential for developing an effective therapeutic agent. A number of vector systems have been described in the literature for producing recombinant antibodies *in vitro*. For An example is shown below. The advantage of this vector is that the antibody heavy and light chain genes are on a single plasmid as opposed to two separate plasmids, thereby simplifying the cell transformation and clone selection process. The steps to convert an scFv to whole IgG are relatively straightforward and involve re-cloning the VH and VL domains of the scFv into the appropriate sites of the IgG expression vector (see diagram below) followed by transfection into Chinese hamster ovary (CHO) cells. Once IgG producing clones are identified, they can be grown to a larger scale in stir flasks and the IgG purified from culture supernatants using Protein G chromatography.

Re-characterization of Abzymes: Because the abzyme has been reengineered at this point from an scFv to a whole IgG, its kinetic and stability characteristics my change. Based on previous work, mainly engineering scFvs from whole IgG molecules (Martin et al., 1996), we do not expect the kinetic parameters to change significantly. However, whole IgG stability should be considerably greater than the scFv from which it was derived. Characterization experiments that were initially done to characterize with the scFv will be repeated with the IgG molecule.



4. Future Plans (Phase II and Commercialization)

Biological Studies

Initially, preclinical studies of the therapeutic effectiveness of candidate abzymes will be performed on validated cell and animal models. Further animal studies will focus on the pharmacokinetics, metabolism, and toxicity of the candidate compounds. Animal testing will be performed at an accredited outside contract laboratory such as Bioqual, Inc. in Rockville, MD. The purpose of these studies will be to demonstrate that discovered catalytic antibodies have a beneficial effect in one or more non-human disease models. There are well-established cell and animal models for transplantation and specifically for anti-CD3ε immunotherapies. Moreover, in all cases, these animal models have been used to test (non-catalytic) immunotherapies directed toward the same target molecules that we will be pursuing.

General: An anti-CD3 monoclonal antibody (OKT3) has been on the market for many years for the prevention and treatment of acute allograft rejection. Because of this, cell and animal models of Graft-Versus-Host Disease are well established and well understood. Appropriate control experiments will be carried out in all of the biological studies. In addition, we will do a comparative study with the established therapeutic antibody, OKT3. The hybridoma cell line that produces OKT3 is commercially available (CRL-8000, ATCC, Manassas, VA).

Antibody Production: Milligram quantities of pure antibody will be produced for biological studies. Antibody will be overexpressed in E. coli (scFv) or CHO cells (recombinant IgG) using published protocols. Antibody purification will be by conventional well-known procedures.

Cell Culture Model: For simplicity and low cost, our first choice is to use a published *in vitro* model that uses pooled human peripheral blood mononuclear cells (PBNC)(Cole et al., 1999). This model was used to test a new potentially therapeutic anti-CD3 antibody. The model provided evidence of the immunosuppressive effects of the antibody and showed that it was less mitogenic (released fewer cytokines) than OKT3. We will repeat the well-described experiments exactly as reported in this paper.

Animal Model: If for some reason we are not satisfied with the results using the above *in vitro* model, we will conduct mouse studies with therapeutic abzymes. Animal models of Graft-Versus-Host Disease (GVHD) exist which have been used to test the therapeutic effects of anti-CD3 antibodies (Drobyski et al., 1998; Vallera et al., 1995). In the described aggressive acute GVHD model, mouse bone marrow transplantation precedes the treatment. In the transplantation, the donor bone marrow cells are taken from C57BL/6 mice (available from the National Cancer institute, Frederick, MD) and the recipient mice are H-2 disparate B10.BR mice (Jackson laboratory, Bar Harbor, ME). The transplantation protocol is described in detail in the literature. The median survival time for untreated mice receiving the transplant is 20 days. Beginning 7 days after the transplant, abzyme and antibiotic (either Cefoxitin or Cefotaxime) will be injected using various reasonable dosing and timing regimens. The regimens will depend on a number of pre-determined factors including the pharmacokinetics (blood level vs. time profile) and tolerance of the antibiotic and abzyme. A beneficial therapeutic effect will be regarded as a median survival time of longer than 80 days.

All reagents, cell lines, and animals that are required for these studies are commercially available at reasonable cost. Appropriate control experiments will be carried out, including administration of an irrelevant antibody in place of the * catalytic antibody, administration of antibiotic alone, and administration of buffer alone. In addition, for all animal studies we will routinely run serum chemistries, complete blood panels, and safety screening. Pharmacokinetics (*ex vivo* or *in vivo*) and tolerance studies will be performed on groups of 3 mice. Catalytic antibody efficacy testing (and related control experiments) will be performed in replicates of 6-10 mice.

Clinical Trials, Manufacturing and Marketing

After completion of the abzyme discovery and cell/animal testing stages, Phase I and Phase II human clinical studies will be pursued in collaboration with prominent academic centers. Before beginning Phase I trials, an Investigational New Drug (IND) application will also be filed with the FDA describing all preclinical results as well as preliminary manufacturing standard * operating procedures. After IND approval, Phase I trials will be carried out using 20-80 healthy volunteers to determine safety and dosage. Phase II trials will involve 100-300 patient volunteers to look for efficacy and side effects. Phase III trials will be performed with 1000-3000 patient volunteers to monitor efficacy and side effects (Littlehales, 1999). We will likely partner with a larger biotechnology or pharmaceutical company during Phase III to utilize their financial and organizational resources and drug development expertise. In return for their support during Phase III, partners would obtain marketing rights to the end products. Phase III results will form the basis of our application for regulatory approval from the FDA.

Small-scale production of antibodies for Phase I and Phase II trials will be outsourced to a GMP-approved, contract antibody manufacturer. Possible candidate companies are Covance (Research Triangle Park, NC) and Bio Science Contract * Production Corp. (Baltimore, MD). Process development will be carried out at IGEN by biochemists and process chemists. Larger scale production of antibodies for Phase III trials and initial sales will be carried out by a contract manufacturer. Depending on the commercial success of the product, IGEN would then decide whether to build its own therapeutic catAb manufacturing facility.

There are two significant advantages to building one's own manufacturing facility: (1) the ability to control production costs by using dedicated facilities and negotiating raw material prices; and (2) independence from companies who control the supply of your product. The primary advantages of outsourcing, on the other hand, are not having to invest in building a facility * and hiring experienced staff and minimizing one's financial risk if sales drop. The ultimate decision about whether to build our own facility will depend on production volume at which our anticipated revenues will equal the sum of fixed and variable production costs (i.e., the break even volume) and the anticipated new product pipeline that would keep the plant running.

With respect to opportunities for strategic alliances, we note that three companies, Taisho Pharmaceuticals, Celltech, and MedImmune have expressed an interest in our catAb technology. Taisho is one of the top ten pharmaceutical companies in Japan, with revenues of $1.9 billion in 1998. Immunological and allergic disorders (including RA and asthma) constitute a strategic R&D focus for the company. Celltech is one of the largest companies in the European biopharmaceutical sector, with revenues * approaching $1 billion in 2000. The company is aggressively developing therapeutic antibodies, and has a mAb against TNFα in clinical trials. Based in the United Kingdom, Celltech also has operations in the United States. MedImmune is a biopharmaceutical company with three approved therapeutic antibodies on the market for treating infectious diseases and a pipeline of therapeutic proteins for treating transplant rejection, cancer and autoimmune diseases. A letter of support from MedImmune expressing interest in our catAb technology is appended (Appendix 1).

5. Risk Factors

Technical Risks The technical risks involved in developing therapeutic abzymes fall into two broad categories: 1) risks in discovering abzymes, and 2) risks involved in the abzymes being therapeutically effective.

Abzyme Discovery Risks: There are no guaranteed methods of making catalytic antibodies. Often, attempts to make abzymes using conventional methods fail for unknown reasons. We will depart from established methods to develop abzymes by * two new and risky approaches – directed evolution and HTS. Although we have technical expertise in all of the steps of the new methods, we have not yet attempted directed evolution or HTS methods to discover abzymes.

Another risk is the chemical reaction to be catalyzed by the abzymes. We plan to inactivate target proteins by acylation of target proteins with β-lactam antibiotics - a reaction that has never been catalyzed by an antibody or enzyme. Feasibility studies * have shown that the uncatalyzed reaction occurs at a reasonably rapid rate, suggesting that an abzyme may catalyze the reaction. We have thoughtfully considered all of the technical details and believe them to be coherent and logical based on published literature and personal experience in catalytic antibodies.

There are a couple of CD3ε-specific uncertainties. Firstly, it is not clear if any of the five extracellular lysines are by themselves critical for T-cell activation. They are spaced widely in the amino acid sequence, increasing the probability * that at least one is essential (Elgert, 1996). On the other hand, only one of the five lysines is conserved among mammals. A deletion mutant of that lysine binds more weakly to the δ subunit than does the wild type protein (Borroto et al., 1998). Secondly, it appears that CD3ε naturally undergoes conformational changes. In some cases, the binding of anti-CD3ε antibodies depends highly on the conformation of CD3ε (Salmeron et al., 1991). Problems could arise if CD3ε is particularly flexible. For example, does the conformation of expressed (solution phase or solid phase) CD3ε differ from the native state conformation?

Therapeutic effectiveness risks: If we discover catalytic antibodies, they may not be therapeutically effective in humans. The abzymes must not only be able to catalyze chemical modification of a target protein, but they must do it with sufficient specificity and speed to have a therapeutic effect. The abzyme must modify an amino acid sidechain on the surface of the target * that is essential for biological activity. The abzyme must also pass through human clinical trials before being approved as a therapeutic. A number of risks are involved in such trials. These risks are high and not limited to abzyme therapies, but are common to all candidate drugs. Moreover, for all chosen targets, there are competitive non-catalytic immunotherapies under development or on the market. The therapeutic abzymes that we develop must be safe, stable, and kinetically efficient to compete in the medical marketplace.

Note Regarding Technical Risks: Although the combined risks of the proposed work are high, through careful planning we have reduced them as much as possible. The new methods of creating catalytic antibodies were designed to streamline the * abzyme discovery process and increase the discovery success rate. The methods substantially reduce the time and manpower required to produce abzymes, allowing more attempts in a given time period. Moreover, because the methods are laborsaving, during the funding period we can use out both directed evolution and HTS approaches with two different antibiotic substrates, Cefoxitin and Cefotaxime. This multi-faceted approach substantially reduces the overall risk.

Commercialization Risks

Perhaps the most significant post-approval, commercial risk associated with abzymes relates to competition from existing, FDA approved mAbs that have already begun to capture market share. As noted above, we believe that existing commercial mAbs for transplant rejection will help to accelerate the market penetration of a therapeutic abzyme. Also, our versatile discovery platform will enable us to identify candidate abzymes against other targets associated with transplant rejection.

Another commercial risk relates to manufacturing the catalytic antibody. As discussed above, the decision about whether to outsource or manufacture in-house will depend on both financial and strategic considerations.

The lifetimes of our existing patents on catalytic antibodies constitute another risk factor. Our earliest patents expire in 2008, which is earlier than we would expect to have an approved drug on the market. On the other hand, our portfolio is structured in such a way that catAb technology is covered by several tiers of increasingly more specific patents, some of which have not yet issued.

Another risk factor relates to our ability to develop and effectively manage strategic alliances. Given our lack of in-house expertise in pharmaceutical marketing, we will likely team up with one or more biopharmaceutical firms to market therapeutic abzymes. If these partners do not devote sufficient resources towards this goal, our opportunities for success will be limited.

A potential long-term market risk is physician reluctance to prescribe a radically new type of immunotherapy such as catalytic antibodies. We believe that this risk can be mitigated through extensive clinical testing and educational marketing. In addition, the increasing use of therapeutic mAbs is changing attitudes towards biological therapeutics in general.

We would be adversely affected by third party payer reluctance to reimburse for therapeutic abzymes. This argues for our initially marketing therapies for diseases that already have approved and reimbursed mAb therapies. In such cases, we would not expect opposition from insurance companies since a therapeutic abzyme would be significantly more cost effective than a mAb.

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APPENDIX 1: LETTER OF SUPPORT

Biochemistry (Moscow), Vol. 65, No. 11, 2000, pp. 1233-1244. Translated from Biokhimiya, Vol. 65, No. 11, 2000, pp. 1459-1472.

REVIEW

Catalytic Antibodies (Abzymes) Induced by Stable Transition-State Analogs

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Received April 10, 2000
Revision received June 5, 2000

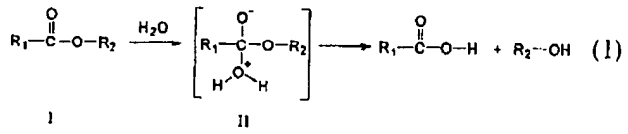
Abstract—This review deals with recent advances in the generation of catalytic antibodies by the immunization of animals with stable transition-state analogs. Characteristic features in the functioning of such abzymes are considered in comparison with traditional enzymes.

Key words: catalytic antibodies, stable transition-state analogs

Binding of immunoglobulins (Ig) to various compounds is a basic and the most studied property of these proteins. The structure, specificity, and mechanisms of functioning of immunoglobulins have been considered in several reviews [1-3].

Antibodies bind specific antigens with high affinity, and this specific interaction includes the formation of numerous noncovalent hydrophobic, electrostatic, and van der Waals forces and hydrogen bonds. The remarkable similarity between the specific action of antibodies and enzymes was noted by Pauling in 1948; he suggested [4] the possibility of immunoglobulins possessing enzymatic functions. However, direct experimental evidence of this possibility was obtained only several decades later.

Enzymes accelerate rates of chemical reactions by 6-10 orders of magnitude [5, 6]. They specifically bind one or a few substrates by forming specific noncovalent bonds. The main mechanisms of enzymatic catalysis utilizing binding energy for the acceleration of the reaction rate have been determined; the results gave rise to the theoretical "transition-state" model of enzymatic reactions, the transition state being stabilized by the enzyme and characterized by increased energy level compared with stable compounds involved in the reaction [5, 6]. For example, hydrolysis of esters in which a carbon atom is bound to three other atoms (I) is accompanied by the formation (after addition of a water molecule) of an unstable transition state (II) characterized by the tetrahedral state of the carbon atom:



(1)

Such transition states of chemical reactions exist during a very short time (fractions of a second or less) and are converted into stable products of lower energy [5, 6].

According to classical concepts in immunology, antibodies can bind only stable ligands or antigens, whereas enzymes can "fix" the high-energy states of transition complexes. This implied the existence of principally different mechanisms of action of antibodies and enzymes. However, results of subsequent studies led to revision of dogmas on the nature of specific binding of ligands with antibodies and the genetic reasons for remarkable variability of the antigen-binding regions of Ig. More detailed studies have revealed many similarities between the interaction of antibody with its complementary antigen and enzyme with the transition state.

When an antigen acts as a ligand, the antibodies insignificantly change their conformation, so in most cases antigen–antibody interaction follows the "lock and key" model initially described for enzymes [7, 8]. However, antibodies (as well as enzymes) are conformationally active molecules. Their interaction with ligands may be accompanied by conformational changes, which can vary from small shifts of side chains of amino acid residues to global changes of tertiary and quaternary structures of Fab fragments of Ig molecules [9-11]. For example, structural complementarity between an antibody and a ligand, which is either structurally related to

Abbreviations: AB) antibody; AG) antigen; Ig) immunoglobulins; TSA) transition-state analogs.

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(antibody cross-reactivity) or distinct from the antigen (antibody polyreactivity), can be achieved by conformational changes of both the antibody and the ligand [12]. Thus, antibody interaction with ligands may include elements of the induced fit mechanism (and substrate deformation) typical for enzyme–substrate interaction [13].

As in the case of enzymes, water molecules play an important role in the formation of antibody–antigen complexes. In complexes of antibodies with protein antigens, water molecules can be located not only on the periphery but also in the region forming contact between antibody and antigen [14]. Water molecules have been recognized in contact regions of antigen complexes with anti-peptide, anti-DNA, and anti-hapten antibodies. Moreover, water molecules can be a required element forming structural complementarity between antibody and antigen [15].

There are many similarities between the formation of the active sites of enzymes and the "active sites" of antibodies. Formation of the antigen-binding site on an antibody molecule involves amino acid residues of the complementarity-determining region; however, not all amino acids of the hypervariable regions make direct contact with the ligand. Most of them are responsible for formation of a unique conformation of the antigen-binding site needed for ligand binding. Amino acids of framework regions in the hypervariable parts of antibodies are also involved in the formation of the antigen-binding site; they can make direct contact with the antigen [1]. Recently, the amino acid composition of antigen-binding sites has been characterized. The frequencies of asparagine and histidine residues are from two to eight times higher in the complementarity-determining region than in the framework region [16]. The antigen-neighboring region is characterized by high frequencies of tyrosine and tryptophan residues [17]. This was also noted for most enzymes that bind nucleotides, DNA, RNA, and other substrates containing hydrophobic and aromatic or heterocyclic groups [5, 6, 18-23].

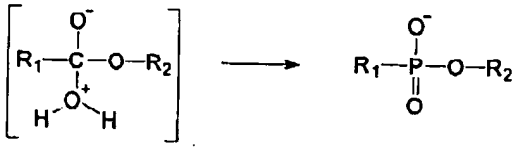
X-Ray analysis has revealed many similarities in structural features of active sites of various enzymes binding structurally related ligands and significant differences when enzymes interact with distinct substrates of various structure and molecular mass [18-23]. Results of numerous studies of antibody–antigen complexes reveal several types of Ig antigen-binding site structures (which could be also found in enzymes): a) cavities, typical for low-molecular-weight ligand-binding sites (hapten-binding sites); b) grooves, formed in the case of peptide-, DNA-, or polysaccharide-binding sites; c) planar regions formed by the active sites of protein-interacting antibodies [9]. However, some exceptions from the "specificity" of these antigen-binding site structures also exist. For example, hapten-binding site can be groove-shaped, as in the case of peptide- and DNA-binding sites [24].

These data on some similarity in the structure of ligand-binding sites, conformation lability of enzyme and antibody active sites, inducibility of conformational changes in protein structure of antibody by antigen and in enzyme by substrate (and vice versa) together with data on antigen and substrate susceptibility to water molecules suggest the existence of some "functional similarity" between antibodies and enzymes. This also suggests certain similarity between the interaction of antibody with complementary antigen and enzyme with transition state.

In 1969 Jencks, taking into consideration maximal complementarity of the active sites of enzymes to transition states of the chemical reaction rather than to the substrates themselves, proposed a hypothesis that antibodies obtained during immunization with a stable transition-state analog could catalyze these reactions, and active sites of these antibodies may contain structural elements similar to that of enzymes [7].

This theoretical parallelism stimulated directed design of antibodies that could catalyze chemical reactions. In the first attempt to obtain antibody-enzymes, Reso and Stollar [25] immunized rabbits with protein conjugate with N-(5-phosphopyridoxyl)-3'-aminotyrosine. However, the resulting antibodies only weakly accelerated the reaction of tyrosine transamination [26]. The first antibody-enzymes, or **ABZYMES** (**Anti**Body-en**ZYME**) were simultaneously and independently obtained in 1986 by two research groups, R. Lerner [27] and P. Schultz [28], and so in the review [29] it was stated "catalytic antibodies were born in California".

Abzymes with esterase activity were obtained using hybridoma technology and immunization of animals with a stable transition-state analog followed by selection of B-lymphocyte clones capable of generating antibodies with designed catalytic properties [27]. A phosphorus atom was used for the modeling of the transition-state of a tetrahedral carbon atom; phosphoric acid ester satisfactory modeled the transition-state in the reaction of carboxylic acid ester hydrolysis:



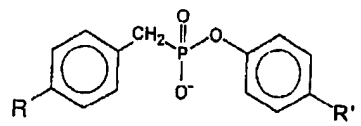
(2)

II
transition-state

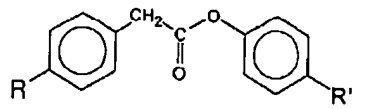
III
transition-state analog

Monoclonal antibodies induced by phosphoric acid ester were able to catalyze hydrolysis of esters. Using similar strategy, antibodies hydrolyzing carbonate were obtained [28]. For the design of such abzymes and also antibodies possessing amidase activity [30], haptens were used [30]; the hydrolyzing bond (C–O or C–N) typical

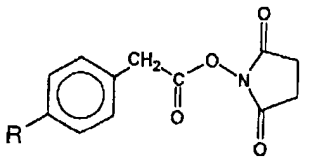
for substrates was substituted in them by the P–O or P–N bond [29, 31-41]. Nevertheless, these and other abzymes obtained in the first stage of the development of abzymology were characterized by low catalytic rates and specificity. Optimal hapten preparation required maximal similarity between structure of the employed analog and the transition-state structure: it should maximally differ from the initial substrate structure. The above-mentioned studies became a basis for the design of transition-state analogs (TSA) for the generation of monoclonal antibodies with designed catalytic activities. Several reviews deal with various problems of abzymology and their solutions at various stages of the development of this field [29, 31-41].



IV



V (3)



VI

R = $NHCOCF_3$, R' = $NHCOCH_3$,

CURRENT PROBLEMS IN THE DESIGN OF EFFECTIVE MONOCLONAL ABZYMES

Design of highly specific monoclonal antibodies with high specific activity and affinity to substrates represents one of the main problems of modern abzymology. This is a very difficult task. Values of maximal velocities and dissociation constants (or Michaelis constants) are linked by inversed dependence: the higher affinity of the enzyme for its substrate, the lower the rate of its conversion [5, 6, 18, 19]. So, almost all highly active enzymes of living organisms have relatively low affinity for their substrates (0.01-1 mM) [5, 6]. High catalytic activity of enzymes with high affinity for their substrates is usually achieved only by energy supply during hydrolysis of macroergic bonds of some cofactor, e.g., ATP [18-20]. In most cases, antibodies have quite high affinity for antigens (0.1-10 μM), so high rates of chemical reactions are basically unachievable. Thus, too high affinity of abzymes for their substrates may seriously complicate the design of effective biocatalysts.

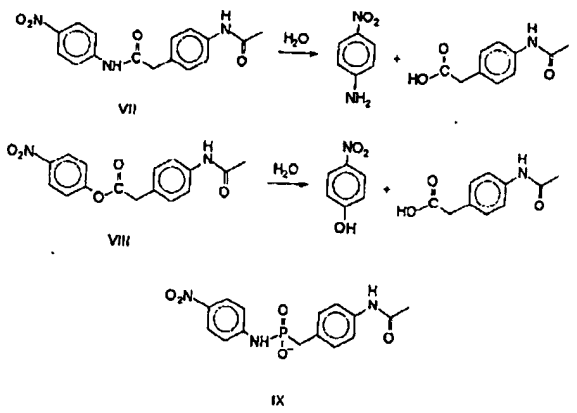
Design of TSA and their protein adducts is the other problem for the development of highly specific and effective abzymes. At present, antibodies hydrolyzing ester or amide bonds represent the main proportion of the numerous monoclonal abzymes. Design of TSA of hydrolytic reactions proceeding via H_2O-dependent intermediate formation of a partially charged tetrahedral transition-state of carbon atom (1) usually includes derivatives of a tetrahedral phosphorus atom (2) [27-41]. Tramontano et al. used a series of TSA for immunization [27]. The best results were obtained with compound IV (3). In the phosphonate group (IV), the spatial location of ligands at the phosphorus atom corresponds to that of substituents at the carbon atom of substrates (compounds V and VI) [27].

Monoclonal antibodies 6D4 for hapten IV hydrolyzed ester V with $K_m = 1.9 \cdot 10^{-6}$ M and $k_{cat} = 2.7 \cdot 10^{-2}$ sec^{-1}. The acceleration of the reaction rate (versus non-catalytic hydrolysis of this ester) was 960-fold:

The hydrolysis of ester VI catalyzed by these abzymes proceeded at a lower rate ($k_{cat} = 8 \cdot 10^{-3}$ sec^{-1}, $K_m = 6.2 \cdot 10^{-7}$ M). During interaction with substrate, antibodies with high affinity for hapten, TSA IV, lowered activation energy for the reaction of ester hydrolysis [27]. For comparison of efficacy and specificity of abzymes as biocatalysts, the authors proposed to use k_{cat}/K_m. In traditional enzymology, the latter is a commonly used parameter representing a measure of the kinetic energy barrier for the enzymatic reaction. It is limited by a value 10^9 $M^{-1} \cdot sec^{-1}$ when mutual diffusion of substrate and enzyme becomes a rate-limiting stage of the reaction. The ratio of rate constant of the enzyme–substrate complex conversion to product (k_{cat}) to rate constant of substrate conversion into product in the absence of catalyst (k_{uncat}) is the other useful criterion. Maximal k_{cat}/k_{uncat} values for enzymatic reactions reach 10^{10}-10^{12} [5, 6].

Lerner's group continued the design of highly effective biocatalysts by employing stable TSAs for immunization. For improvement of catalytic properties of abzymes Tramontano et al. [42] selected monoclonal antibodies with the highest affinity for TSA (IV (3); R' = $NHCO(CH_2)_4CON(COCH_2)_2$). Monoclonal IgG, products of clones 50D8 and 57G4 catalyzing hydrolysis of ester V (R' = $NHCOCH_3$ (3)), have been obtained. The activity of antibodies 50D8 was comparable to that of known esterases: the k_{cat} value was only 10-100 times less than that of pig liver esterase catalyzing the hydrolysis of an ester similar to ester V. Moreover, the k_{cat} value for antibodies 50D8 was 10^2-10^4 times higher than that of chymotrypsin in the reaction of hydrolysis of synthetic substrates; it was within a range of k_{cat} values for most other esterases. The k_{cat}/k_{uncat} parameter for 50D8 abzymes was ~10^6. The significant improvement of 50D8 catalytic properties versus 6D4 abzymes was achieved by the high affinity of these antibodies to the TSA ($K_i = 50$ nM), but their affinity to substrate V was quite low: $K_m \sim 1.5$ mM.

For the generation of antibodies catalyzing amine hydrolysis, Schultz's group [28] used phosphonamide TSA IX:



VII

VIII (4)

IX

After immunization with conjugate of this hapten with BSA, monoclonal antibodies were obtained. Based on the ELISA method for detection of TSA binding to abzymes, 44 clones of B-lymphocytes were selected. Products of only one clone, 43C9, hydrolyzed amide VII (4), and the values of the kinetic parameters were as follows: $k_{cat}/k_{uncat} = 1.5 \cdot 10^5$, $k_{cat}/K_m = 2.24$ $M^{-1} \cdot sec^{-1}$. Besides hydrolysis of *p*-nitroanilide derivative VII, abzymes 43C9 also catalyzed hydrolysis of *p*-nitrophenyl derivative VIII. The latter process was characterized by the high (for abzymes) k_{cat}/K_m ratio of $1.5 \cdot 10^5$ $M^{-1} \cdot sec^{-1}$ and one of the highest k_{cat} values (40 sec^{-1}). Abzymes 43C9 with the best esterase properties were obtained and selected using TSA IX of the reaction of hydrolysis of amide VII (4) [31]. This suggests some unpredictability of the immune system in generating catalytic antibodies.

Studying possibilities of induction by one TSA of several types of abzymes that catalyze reactions via various mechanisms, different authors used "single-chain" antibodies from phage libraries of light and heavy chains [43, 44]. For the development of such constructions, cDNA copies of hybridoma 43C9 total mRNA were inserted into phage λ, and then phages expressing heavy or light chains of Ig were selected [45]. Individual phage libraries of heavy and light chains were crossed to get recombinant libraries. They were screened by expression of high affinity antibodies for the hapten, TSA. The selected gene-engineered phage antibodies exhibited catalytic properties similar to that of products of the initial hybridoma 43C9. Using the abzyme 43C9 heavy chain gene and the phage library of *statistical set* of light chain genes, several constructions of "single-chain" antibodies with esterase activity have also been obtained [44]. DNA analysis of light chains of this abzyme family exhibiting the same activity as abzymes 43C9 revealed high homology within this family and 96-98% homology with antibody 43C9 light chain. All abzyme light chains have conserved ArgL96 and HisL91 and their nearest amino acid environment. All members of this abzyme family were characterized by very similar values of parameter k_{cat}/k_{uncat}. This suggests that during formation of immune response against TSA-hapten the abzymes of the 43C9 family have optimal affinity and the best catalytic characteristics. Subsequent improvement of catalytic properties of antibodies requires some changes in the conserved sites of variable regions. At present, such changes can be achieved using gene-engineering constructions rather than immunization and hybridoma technology. Miller et al. [44] analyzed relative limitations for the employment of immunization with TSA-haptens for design of enzymes with desired properties. The structure of the antibody active site is believed to be the first "template", which opens the possibility for subsequent modernization of antibodies to obtain more effective and specific biocatalysts.

Detailed study of hydrolysis of esters and amides catalyzed by abzymes has revealed some common features that are also typical for mechanisms of other reactions catalyzed by monoclonal antibodies against TSAs. First, the effect of structural resemblance between TSA-substrate and reaction products should be mentioned. Functional groups of the substrate modified during its catalytic conversion cannot provide high affinity of monoclonal antibodies for the transition-state. Constant, unchanged groups of the substrate make a substantial contribution to the binding energy [46]. So, dissociation of the enzyme–product complex often becomes the limiting stage of the catalytic conversion; end-product inhibition may occur. It is suggested that gene engineering of abzymes may overcome this shortcoming [47].

Heavy chain amino acid residues make the greatest contribution to substrate binding by antibodies of the 43C9 family, whereas light chain amino acid residues are involved in stabilization of the transition state of the reaction, i.e., they directly participate in the catalytic stage [48]. In most natural and hybridoma-derived abzymes, light chain amino acid residues play a decisive role in the catalysis. Basic studies of antibody–antigen interactions suggest that the heavy chain makes a greater contribution to antigen recognition than the light chain [49]; the latter can probably explain the greater "catalytic potential" of the light subunit of the Ig oligomer. It is possible that light chain structure is responsible for the realization of the particular catalytic mechanism of the reaction.

Design and studies of abzymes with esterase and amidase activities represent only a small proportion of the vast number of publications dealing with methods of generation of catalytic antibodies (see for review [40, 50]). At present, more than 100 reactions catalyzed by monoclonal abzymes obtained by immunization with various haptens, stable TSA, and by other approaches are known. The table summarizes examples of some haptens used for immunization and substrates of reactions catalyzed by the monoclonal abzymes. These data suggest that design of hapten allows antibodies responsible for stereo-specific catalysis of reactions with substrates and products, which

Haptens used for immunization and substrates and products of reactions catalyzed by the monoclonal antibodies

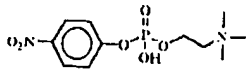
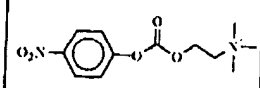
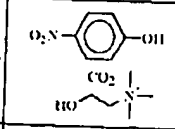
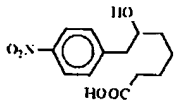
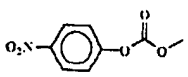
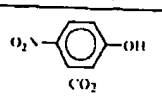
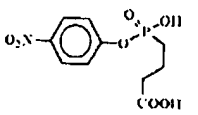
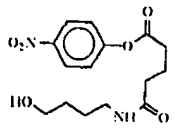
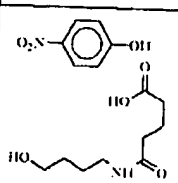
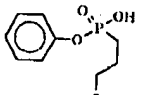
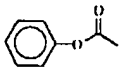
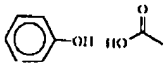
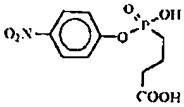
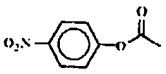
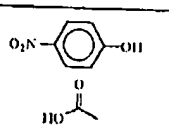
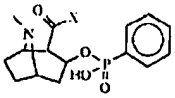
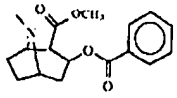
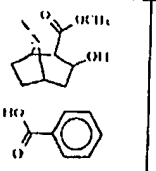
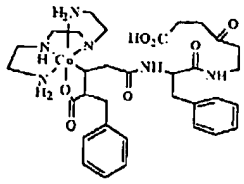
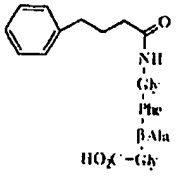
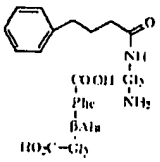
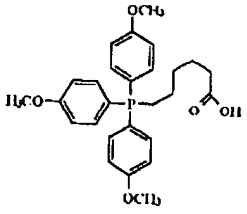
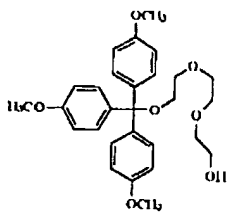
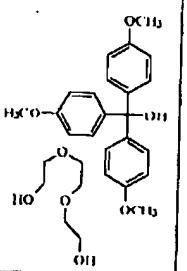
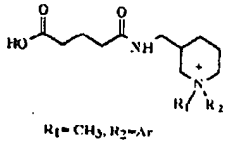
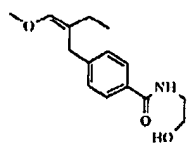
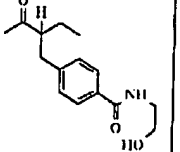
Hapten	Substrate	Products	Antibodies (conditions of reaction)	K_m, μM	k_{cat}, sec^{-1}	k_{cat}/K_m, $M^{-1} \cdot sec^{-1}$	References
1	2	3	4	5	6	7	8
Carbonate hydrolysis 			MOPC 167 pH 7.0, 30°C	208	0.007	3.36	[28]
			7K16.2 pH 7.5, 30°C	3330	$5.2 \cdot 10^{-3}$	1.56	[34]
Ester hydrolysis 			CNJ157 pH 8.0	110	0.040	364	[51]
			20G9 pH 8.8, 25°C	300	0.152	500	[52]
			KD2-260 pH 6.0, 20°C	4.9	0.042	8600	[53]
 X=(CH2)3NHCOCH2CO2H			3B9 pH 7.7	490	0.0018	3.74	[54]
Amide hydrolysis 	 Gly Phe β-Ala HO2C–Gly	 COOH Gly Phe NH2 β-Ala HO2C–Gly	28F11 pH 6.5, 37°C	—	$6 \cdot 10^{-4}$	—	[55]
Ether hydrolysis 			37C4 pH 6.0	31	$1.6 \cdot 10^{-3}$	54	[56]
 R_1=CH3, R_2=Ar			14D9 pH 5.7, 37°C	340	$9.5 \cdot 10^{-5}$	0.279	[57]

Table (Contd.)

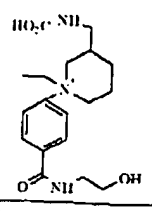
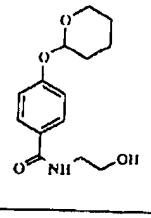
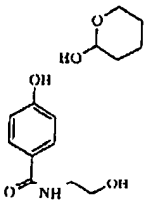
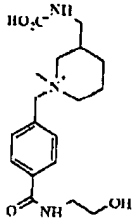
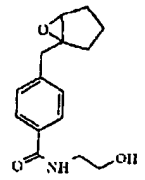
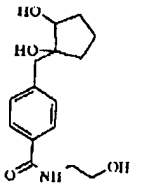
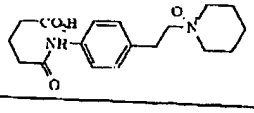
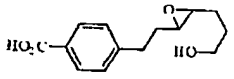
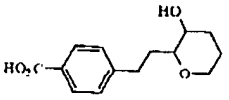
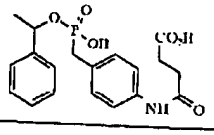
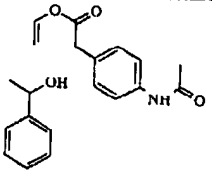
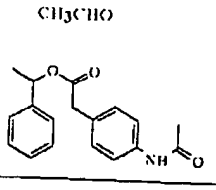
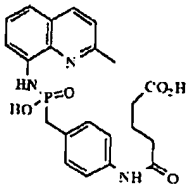
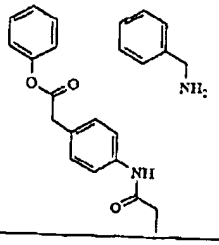
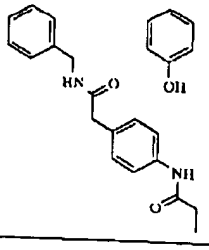
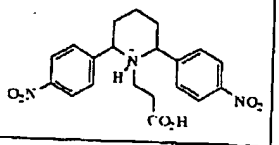
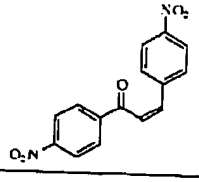
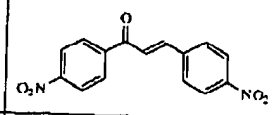
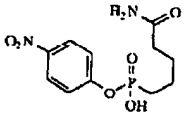
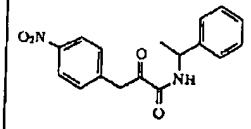
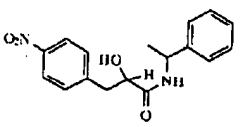
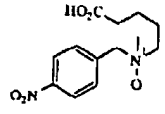
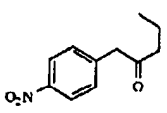
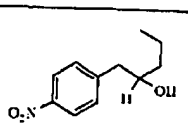
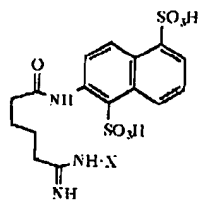
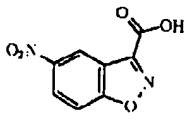
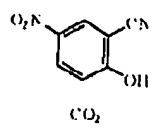
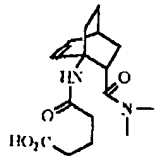
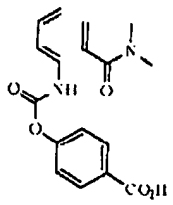
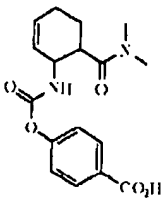
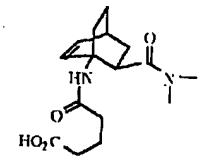
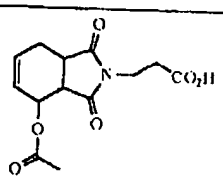
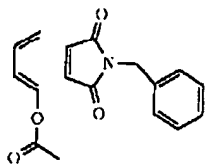
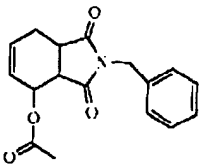
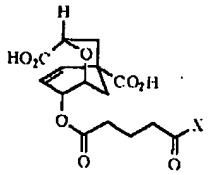
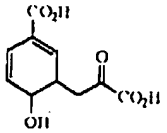
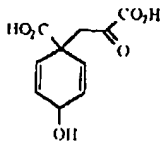
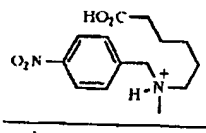
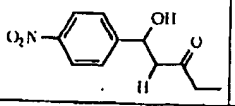
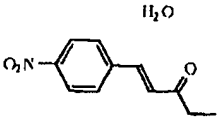
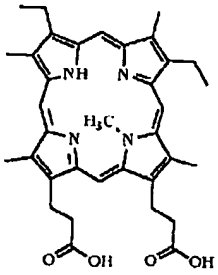
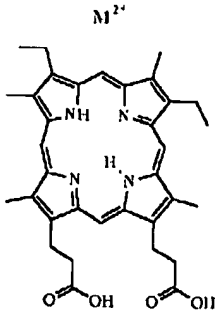
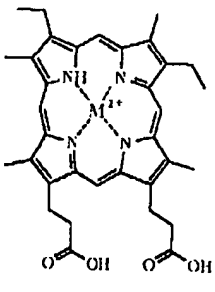
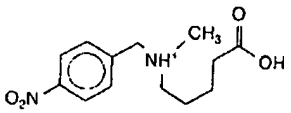
1	2	3	4	5	6	7	8
Ketal hydrolysis				14D9 pH 5.7, 20°C	100	$7.5 \cdot 10^{-5}$	0.78	[35]
Epoxide hydrolysis				14D9 pH 5.6, 24°C	25	$2.9 \cdot 10^{-5}$	1.0	[58]
				26D9 pH 6.6	356	$1.5 \cdot 10^{-3}$	43	[59]
Transesterification				2H6-1	2200	0.067	30	[60]
Amide formation				17G8 pH 8.0, 23°C	2200	$3.8 \cdot 10^{-4}$	0.173	[61]
Isomerization				DYJ10-4 pH 7.5	220	0.08	364	[35]
Reduction				A5 pH 5.0	1240	0.0017	14	[62]
				37B39.3 pH 5.0	52	0.0016	32	[63]

Table (Contd.)

1	2	3	4	5	6	7	8
Decarboxylation 			21D8 pH 8.0, 20°C	168	0.28	1667	[64]
Diels–Alder reaction 			7D4 pH 7.4, 37°C diene dienophile	960 1700	$5.7 \cdot 10^{-5}$	0.06 0.034	[65]
			22C8 pH 7.4, 37°C diene dienophile	700 7500	$5.3 \cdot 10^{-5}$	0.076 0.007	[65]
			H11 dienophile	8300	0.055	6.6	[66]
Claisen regrouping 			1F7 pH 7.5, 14°C	51	$1.2 \cdot 10^{-3}$	24	[67]
Dehydration 			20A2F6 pH 7.0, 37°C	1100	$5.8 \cdot 10^{-6}$	$5.3 \cdot 10^{-3}$	[68]
Interaction of porphyrin with metal 			7G12-A10-G1-A12 Zn^{2+} Cu^{2+}	49 50	$8.6 \cdot 10^{-6}$ $1.4 \cdot 10^{-6}$	0.17 0.028	[69]

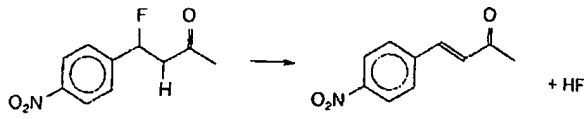
do not have natural analogs, to be obtained. Some antibodies can catalyze reactions via mechanisms that are not known for natural biocatalysts.

One of principally new approaches for the construction of haptens for the generation of abzymes with designed properties was proposed by Shokat et al. [70]. Using insertion of specific immunogenic groups into the hapten, the authors tried to generate a set of amino acids at the antibody active site that are typical for active sites of enzymes. A positively charged (at physiological pH) quaternary ammonia base was inserted into nitrophenyl-derivative X (5). These authors suggested that this would allow the generation of carboxyl-containing amino acid residues (glutamate or aspartate) at the active site of abzymes that accommodates this hapten.



X

(5)



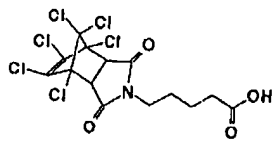
XI

According to their consideration [70], a carboxyl group at the active site would operate as an intermediary proton acceptor, as Asp and Glu residues do operate in the hydrophobic environment of the active sites of some enzymes. The resultant monoclonal abzymes 43D4-3D3 against hapten X actually catalyzed the reaction of β-elimination given above (5).

The ratio k_{cat}/K_m for this reaction was $1.38 \cdot 10^2$ $M^{-1} \cdot sec^{-1}$, and these abzymes accelerated the reaction rate by three orders of magnitude compared with the non-catalytic process.

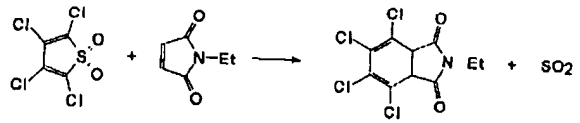
Study of kinetic properties of this conversion and inhibition of abzyme activity by specific covalent modification of carboxyl residues suggested the involvement of either Glu or Asp residues in the catalysis. This confirmed the effectiveness of the proposed approach for the hapten selection. It should be noted that this hapten did not resemble a TSA. Moreover, the charge of this hapten was opposite to that of the transition state. This approach [70] was subsequently adapted for the design of antibodies catalyzing dehydration [68] and *cis-trans*-isomerization [35] reactions (table).

For the construction of transition-state analogs of the Diels–Alder reaction, Na et al. [71] relied on a well-studied mechanism of reaction involving N-substituted maleimide XIV:



XII

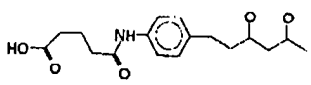
(6)



XIII XIV

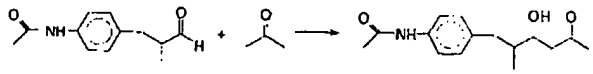
Monoclonal antibodies 1E9 bound TSA XII with K_d $1.3 \cdot 10^{-6}$ M and accelerated bi-substrate cyclization reaction (6) by ~100-fold. Subsequently, this approach was used for preparation of abzymes catalyzing stereospecific condensation via the Diels–Alder mechanism [65]. In the considered examples, abzymes catalyzed the reactions via mechanisms that are not realized by natural biocatalysts. This also demonstrates the promise for the use of TSA-haptens for generation of abzymes with designed enzymatic activities.

The greatest acceleration of the reaction rate in the presence of abzymes ($k_{cat}/k_{uncat} = 10^9$) was demonstrated using an original approach proposed by Wagner et al. [72]. For generation of antibodies catalyzing acetone condensation with aldehyde XVI, diketone XV was used as the hapten. These authors suggested that active sites of antibodies with the highest affinity for XV would contain a reactive lysine residue:



XV

(7)



XVI XVII

So, antibody interaction with the hapten will include Schiff-base formation. This approach implying generation by hapten of its covalent interaction with antibody was called "reactive immunization" [73].

It was also suggested that an ε-amino group of lysine will form a covalent intermediate with aldehyde XVI, which is converted into reaction product XVII after binding of the second substrate (enolate).

After immunization of mice and elaboration of primary clones of B-lymphocytes, combined phage libraries of genes encoding heavy and light chains of Ig were obtained [74]. Phages were screened for covalent binding to immobilized hapten XV, and thus the phages that display antibodies containing lysine at the active site were

selected. This approach including elements of hybridoma technology and phage display was very effective; it allowed the production of aldolase abzymes demonstrating the greatest acceleration of the reaction rate and 95% stereoselectivity of the catalyzed reaction.

Thus, modern approaches for abzyme production include not only design of hapten and elaboration of monoclonal antibodies against it, but also the preparation of antibody-based gene-engineered constructions and even rational change of the active site by site-directed mutagenesis. Most of the achievements in this field have been patented by their authors. It should be stressed that the practical importance of biocatalysts is a "driving force" for studies of monoclonal abzymes.

COMPARISON OF ACTION MECHANISMS OF ABZYMES AND ENZYMES

Good evidence exists that mechanisms of action of abzymes and enzymes have many similarities.

Study of the mechanism of mixed ester hydrolysis by antibody 43C9 [75] revealed that the catalysis is a multistage process similar to that of peptide hydrolysis by serine proteinases. Data of kinetic analysis suggest that abzymes form a covalent intermediate (Ab^*P_1) with the carboxyl group of the product:

$$\underset{A}{Ab + S} \rightleftharpoons \underset{B}{Ab^*S} \rightleftharpoons \underset{C}{Ab^*I} \xrightarrow{OH^-} \underset{D}{Ab^*P_1^*P_2} \rightleftharpoons \underset{E}{Ab^*P_1 + P_2} \rightleftharpoons Ab + P_1 + P_2 \quad (8)$$

So, as in the case of serine proteinases, the hydrolysis follows the "ping-pong" mechanism. Acceleration of the reaction catalyzed by antibodies 43C9 was achieved not only by stabilization of the transition state but also due to realization of elements of acid–base catalysis. Data of kinetic analysis also suggested that substrate binding is accompanied by significant conformational changes at the active site of abzymes. Consequently, like enzymes, antibodies 43C9 act in accordance with the induced-fit mechanism.

The similarity of TSA antigenic determinants and the substrate required for abzyme generation resulted in formation of reaction product that had high affinity to the active site of the abzymes. So dissociation of the enzyme–product complex was the limiting stage of the hydrolytic reaction (stage E in (8)).

Subsequent studies of the active site of the antibody family 43C9 employed computer modeling of the structure of the abzyme–TSA complex and gene-engineered constructions based on hybridoma 43C9 mRNA as described in [45]. Gene-engineered phage antibodies exhibited catalytic properties similar to that of products of initial hybridoma 43C9. Nucleotide sequences of the DNA copy of antibody 43C9 mRNA and DNA encoding gene-engineered phage-expressed antibodies were identical. Phage constructions of abzyme genes were used for generation of gene-engineered antibodies, NPN43C9 antibodies, in which variable parts of heavy and light chains were linked by a polyglycine spacer (so-called "single-chain antibodies") [76].

Roberts et al. [48] demonstrated that a light chain arginine residue, ArgL96 of antibody NPN43C9 complex with TSA IX (4), is located near the phosphorus atom of the TSA phosphoamide group. This residue is suggested to play key role in the catalysis because it stabilizes the transition state. This hypothesis was confirmed by generating mutant antibodies in which ArgL96 was changed for glutamine [48]. The mutant antibodies were able to bind substrate, but they lacked catalytic activity. Site-directed mutagenesis also revealed an essential role of HisL91 in the catalysis; its imidazole group is suggested [77] to be the nucleophile responsible for intermediate formation with the acyl group of the substrate.

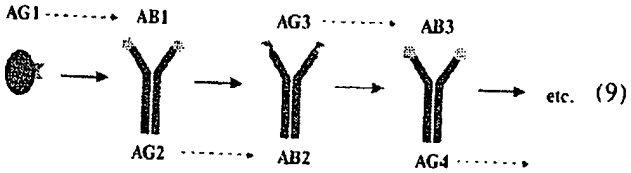
Although data of kinetic experiments [75] suggested the existence of acyl intermediate Ab^*P_1 (8), direct experimental evidence has recently been obtained using mass spectrometry [78]. At saturating substrate concentration ~8% antibodies 43C9 exist as the covalent complex with the product. Mutant HisL91→Gln "single-chain" antibodies can bind substrate but do not form the intermediate compound with it. The authors indicate that the involvement of the imidazole group of histidine in the formation of an acyl intermediate by antibodies 43C9 is unknown for the action of analogous proteinases.

Recently published data of X-ray analysis of TSA complex with abzymes 17E8 possessing esterase and amidase activities revealed that, as in the case of antibodies 43C9, hydrolysis proceeds via the formation of an acyl intermediate [79] and a serine residue acts as the acceptor of the acyl group of the substrate. Interaction with histidine and aspartic acid residues significantly increased the nucleophilic properties of the serine residue at the active site. Thus, the catalytic action of abzymes 17E8 was so close to the catalytic mechanism of serine proteinases that it even "preserved" the catalytic triad Ser-His-Asp typical for the active site of trypsin [79].

TSA-haptens of abzymes 43C9 and 17E8 did not contain structural elements that would lead to the realization of such complex catalytic mechanism. It is possible that the generation of abzymes realizing almost all catalytic elements of natural enzymes with similar activities represents an intrinsic process typical for the immune system. Consequently, the "intension of the immune system" to generate antibodies with maximal affinity and complementarity for hapten results in formation of Ig with structural elements that correspond to the elements selected during evolution for provision of maximal complementarity between the active site of an enzyme and the transition state of a chemical reaction.

OTHER WAYS OF INDUCING ABZYMES

Immunization of animals with various enzymes is another way for the generation of induced abzymes. The development of theoretical abzymology led to the hypothesis of the possibility of abzyme induction based on Jerne's anti-idiotypic network [80]. The simplified model for realization of this network may be presented as follows:



Antibody 1 can effectively bind antigen 1. At the same time, the former can also be an antigen inducing generation of secondary antibody, antibody 2. The latter may also be an antigen causing generation of antibody 3, and this can be continued (antibody 4, antibody 5, etc.). The scheme shows the great similarity between the antigenic determinant of antigen 1 and the antigen-binding site of antibody 2 (the same is true for the couple antibody 1–antibody 3). Antibodies 1 and 2 are denominated as idiotype and anti-idiotype, respectively, etc. There is convincing evidence that such idiotype–anti-idiotype networks are actually present in the body. The presence of blood serum antibody 4 titer (notation of the scheme is used) has been recognized in man and experimental animals [80].

If the active site of an enzyme plays the role of antigen triggering this anti-idiotypic chain, it is logical to suggest that the secondary anti-idiotypic antibody 2 will possess a protein structure, part of which represents a "mould" of the active site of this enzyme and, consequently, these antibodies may possess some properties of this enzyme. It is suggested that DNA-hydrolyzing antibodies generated in patients with systemic lupus erythematosus have anti-idiotypic nature [81, 82]. The authors suggest that these DNA-hydrolyzing antibodies are anti-idiotypic antibodies to topoisomerase I because in blood serum of patients with systemic lupus erythematosus an increased level of antibodies against this enzyme was noted. Data of the other study indicate that DNA-hydrolyzing antibodies are anti-idiotypes to DNAse I [83].

The possibility of generation of anti-idiotypic abzymes as "internal images" of active sites of enzymes was demonstrated by primary immunization of animals with some hydrolytic enzymes and also by primary antibodies to the active sites of these enzymes [84, 85]. It should be noted that abzymes produced using this approach are less studied than the antibodies generated with TSAs.

Apparently, both mechanisms of abzyme induction (antibodies to TSAs and anti-idiotypic ones) are produced in patients with various autoimmune and some viral diseases. Results of studies of natural catalytic antibodies will be considered in the accompanying review [86].

In conclusion it should be noted that recent development of technology for induced abzyme production stimulated not only rapid development of direct approaches for generation of abzymes with designed properties. It also becomes possible to improve abzyme properties by modification of the antigen-binding site of monoclonal antibodies at the level of their genes using methods of selective chemical modification or site-directed mutagenesis. This can be used to obtain abzymes with higher substrate specificity than are found in enzymes. In some cases, rates of chemical reactions catalyzed by abzymes are close to or even higher than that of similar reactions catalyzed by traditional enzymes. Moreover, there are some abzymes that do not have natural enzymatic analogs. For example, they can catalyze the Diels–Alder reaction, which usually proceeds in gas phase at high temperature. This suggests great practical importance of antibodies with designed catalytic specificity in biotechnology and medicine. Production of abzymes catalyzing rapid cleavage of hazardous compounds (including drugs) is one of the promising directions of this field.

This work was supported by the Russian Foundation for Basic Research (grant No. 98-04-49719) and the Siberian Division of the Russian Academy of Sciences (grant for young scientists).

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Catalytic antibodies and other biomimetic catalysts

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Received (in Cambridge, UK) 4th August 2000
First published as an Advance Article on the web 3rd November 2000

Covering: March 1996 to March 2000

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James Stevenson was born in Derbyshire in 1975. He obtained his BSc in Biochemistry and Biological Chemistry from the University of Nottingham in 1996. He then remained at Nottingham to undertake a PhD under the supervision of Neil Thomas where he worked on the development of screening methods for catalytic antibodies. After completing his PhD in 2000 he moved to The Pennsylvania State University to take up a postdoctoral position in Professor Stephen Benkovic's laboratory where his research focuses on the generation of hybrid enzymes using in vitro *evolution techniques.*

DOI: 10.1039/b006389o

1 Introduction

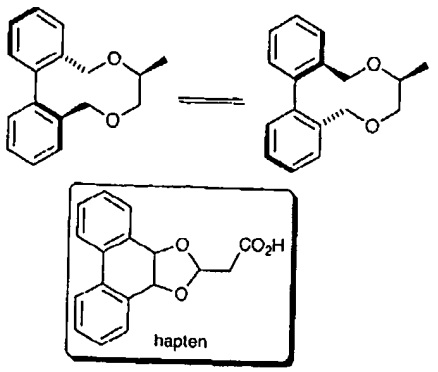
The four-year period since the last review on catalytic antibodies[1] has seen a change in emphasis of this field of research. Whilst a number of new catalytic activities have been reported, as detailed in section two, much of the research reported has focused on understanding the mechanism of action of previously identified catalytic antibodies. This has involved a combination of kinetic and structural biology techniques, with X-ray crystallography playing a central role. The third section of the review details these studies. Improvements in the methods of antibody production through phage display and other methodologies, new methods for screening or selecting for specific catalytic activities, and the optimisation of these activities have also been extensively examined. These are described in sections four and five of the review. Section six describes the exploitation of catalytic antibodies as synthetically useful catalysts, in controlled drug delivery, and in narcotic degradation. Finally section seven contains details of other biomimetic catalysts reported in the last few years and gives a comparison of the efficiency and scope of these different systems. For an introduction to the basis of antibody catalysis, and a historical perspective on the area, the reader is directed to other reviews.[1,2]

2 New catalytic activities

2.1 Isomerisation through antibody-induced strain in the substrate

The simplest type of molecular change for which a catalyst can be generated is that of an isomerisation between two different conformations that are separated by one or more bond rotations. This type of isomerisation is unlikely to be catalysed by acids, bases, nucleophiles or electrophiles, but only through the antibody stabilising the rate-determining transition-state by binding preferentially to it. One of the simplest systems with these isomerisation properties is the bridged biphenyl cyclic ether developed by Mislow and co-workers.[3] Schultz *et al.* have identified seven antibodies generated against a planar hapten structure which catalyse this isomerisation (Scheme 1).[4] The greatest acceleration was seen with antibody 64D8E10 which had a rate enhancement (k_{cat}/k_{uncat}) of 2900. From the activation parameters determined from the Arrhenius plots for the catalysed and uncatalysed reactions the difference in $\Delta G^{\ddagger}$ between the two reactions was ~5 kcal mol^{-1}, mainly due to the enthalpy component. A comparison of K_M/K_i to k_{cat}/k_{uncat} reveals that greater than 80% of the binding energy is converted into catalysis in the case of antibody 64D8E10.

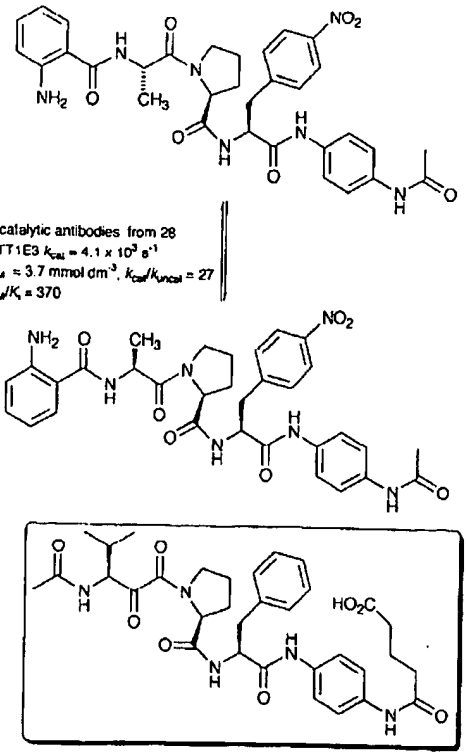
Interestingly an RNA aptamer has also been produced which accelerates this reaction.[5] In this case a rate acceleration 88-fold above background was observed, perhaps reflecting the lower binding affinity the aptamer displays for the substrate/transition-state when compared with the much larger antibody.



7 catalytic antibodies from 32,
Catalytic antibody 64D8E10: $k_{cat} = 4.3 \times 10^{-5}$ s^{-1}, $K_M = 420$ μmol dm^{-3}, $k_{cat}/k_{uncat} = 2900$, $K_d = 210$ nmol dm^{-3}

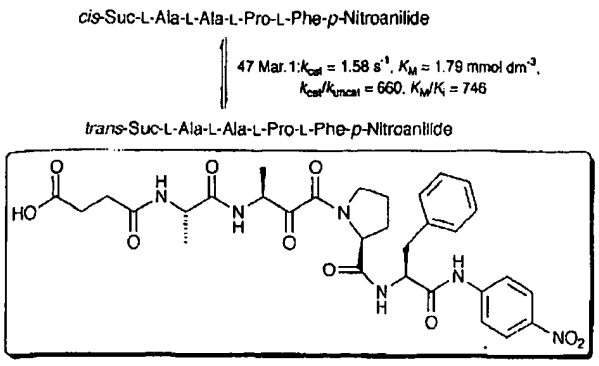
Scheme 1

Rotation around the P_1-proline amide bond is another isomerisation process that has been examined with antibodies. Peptidyl-prolyl isomerases are a group of recently discovered enzymes, which include the cyclophilins and FKBP's (FK506 binding proteins). These enzymes play important roles in protein folding and immuno-regulation. These enzymes are thought to operate through a combination of induced strain and desolvation effects that together overcome the loss of amide nitrogen–carbonyl resonance stabilisation energy in the transition-state. Janda and co-workers have used an α-ketoamide derived from the structure of FK506 to elicit 2 antibodies from a panel of 28 which catalysed the *cis* to *trans* isomerisation of internally quenched fluorescent tripeptides containing a central proline (Scheme 2).[6] The best antibody generated using this approach, VTT1E3 was found to accelerate the reaction 27-fold which is 10^3-fold slower than FKBP and 10^5-fold slower than cyclophilin. More recently Schultz and co-workers have used a similar α-ketoamide hapten to generate antibodies which isomerise both a tetrapeptide and denatured RNase T1 (Scheme 3).[7]



2 catalytic antibodies from 28
VTT1E3 $k_{cat} = 4.1 \times 10^{-3}$ s^{-1}
$K_M = 3.7$ mmol dm^{-3}, $k_{cat}/k_{uncat} = 27$
$K_M/K_i = 370$

Scheme 2



Scheme 3

The ketoamide hapten developed by Janda and co-workers for the proline isomerase reaction has also been found to elicit antibodies that promote a Norrish type II photochemical reaction (Scheme 4).[8] The binding cavity of the antibody promotes the formation of a tetrahydropyrazine product rather than the mixture of oxazolidinone and β-lactam products observed in the absence of the antibody at the same pH.

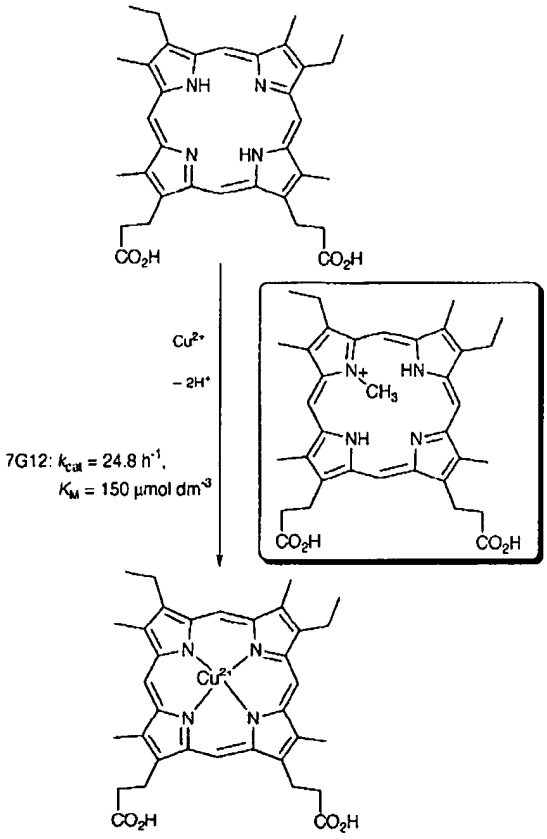
2.2 Metal insertion

Another reaction which primarily requires the antibody to induce strain in its substrate is the cuprochelatase antibody 7G12 which catalyses the insertion of Cu(II) into mesoporphyrin (Scheme 5). This antibody has an efficiency that approaches that of the enzyme ferrochelatase which catalyses the last step in heme biosynthesis.[9] Antibody 7G12 has been crystallised and its structure and mechanism of action is discussed in more detail in section 3.7.

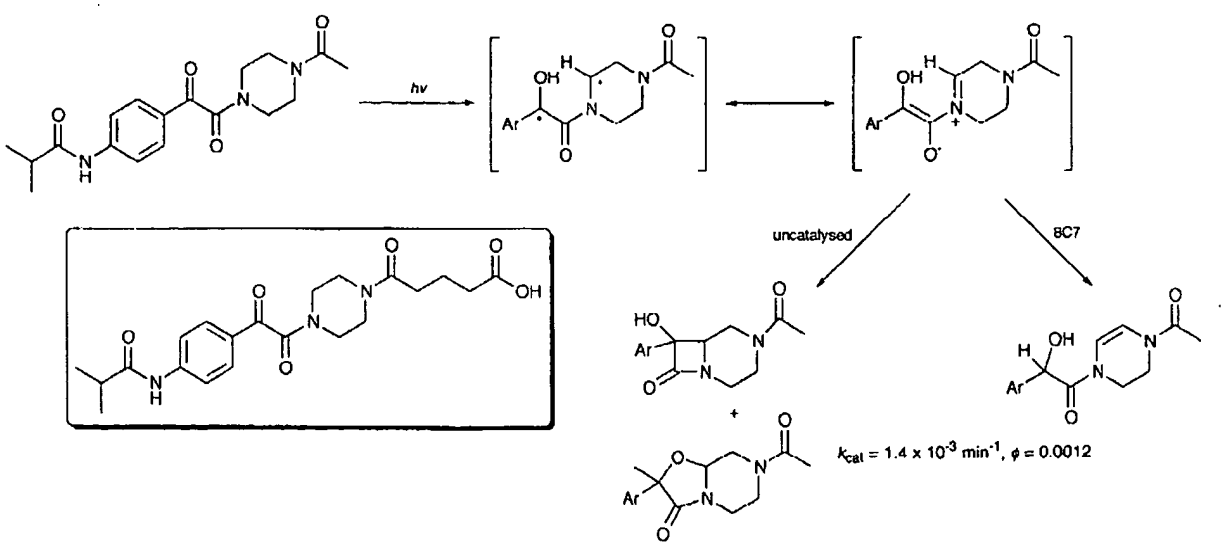
2.3 Proton transfer and catalysis by desolvation

The previous review[1] highlighted the antibody catalysed decomposition of 5-nitrobenzisoxazole to its cyanophenol (the Kemp elimination), and related Kemp decarboxylation reactions. Tellier and co-workers have examined a further hapten based around an amidine structure, for its ability to induce antibodies capable of catalysing the Kemp elimination (Scheme 6).[10] It was found that six of the twenty antibodies which bound this hapten were capable of catalysing the reaction. The two tightest binding antibodies were shown to have the highest catalytic activity. A pH–rate profile for antibody 4B2 indicated that a group with pK_a 5.8 was involved in the reaction, suggesting the presence of a catalytically important aspartate, glutamate or histidine residue. The Kemp elimination has also been catalysed by serum albumins and various synthetic receptors as described in section seven. The same amidine hapten has also been shown to elicit antibodies capable of catalysing the allylic isomerisation of a β,γ-unsaturated ketone to the α,β-isomer.[10] In this case the maximal activity is at pH 4.5 with an aspartate or glutamate sidechain again being implicated, with the reaction passing through a dienol or dienolate intermediate. Multiple turnovers are observed, and the most active antibody 4B2 has been cloned and sequenced with four aspartic acids and one glutamic acid occurring in its hypervariable loops.

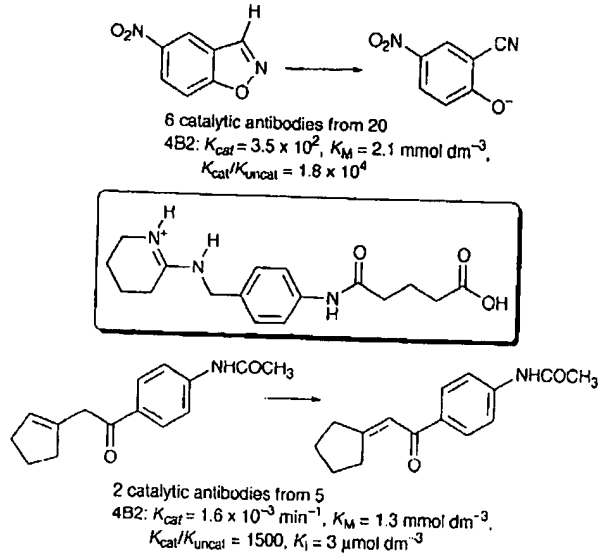
The antibody binding pocket has been exploited in a different manner by Schultz and co-workers in an attempt to control the solvolysis of *endo*-2-norbornyl mesylate.[11] It has been



Scheme 5

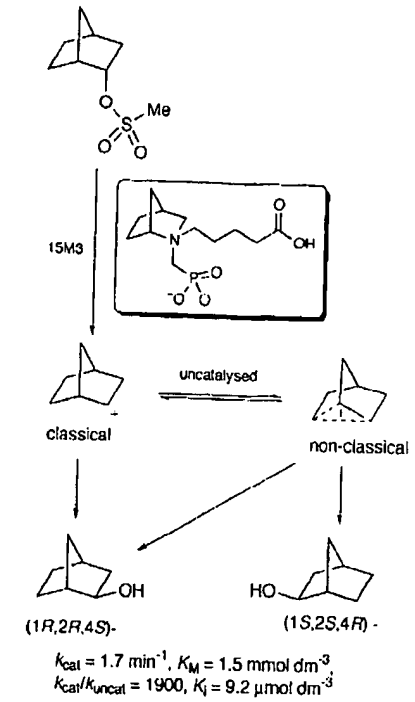


Scheme 4

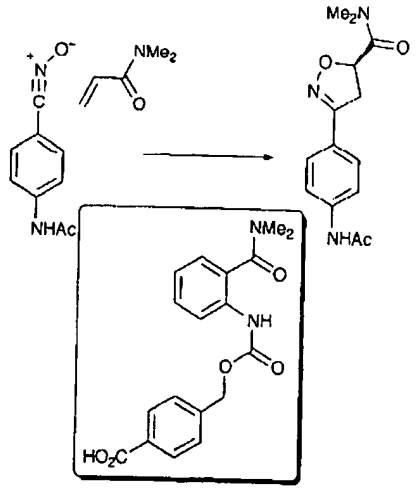


Scheme 6

demonstrated that *endo*-2-norbornyl † mesylate in buffer at pH 7.4 ionises to initially give an unsymmetrical localised cation which is then converted into the more stable non-classical norbornyl cation by participation of the adjacent σ-bond. Calculations predict that the non-classical cation is more stable than the classical cation by ~6 kcal mol^{-1}. Quenching of this cation by solvent generates equal amounts of the homochiral *exo*-norborneols. However in the presence of antibody 15M3 the solvolysis of only one enantiomer, (1*R*,2*S*,4*S*)-*endo*-norbornyl mesylate was enhanced and the resulting product was found to be 96% enantiomerically pure for (1*R*,2*R*,4*S*)-*exo*-2-norborneol (Scheme 7). Further experiments with isotopically labelled substrates laid credence to the reaction occurring *via* a S_N1 rather than S_N2 mechanism in the antibody binding site. Together these facts indicate that the antibody was either able to stabilise the classical cation relative to the delocalised non-classical cation, or the antibody blocks attack at the alternative position on the norbornyl skeleton.



Scheme 7



Scheme 8

2.4 Diels–Alder and other cycloaddition antibodies

Pericyclic reactions catalysed by enzymes have only recently been reported, with the isolation of solanapyrone synthase from *Alternaria solani* which catalyses an enantioselective *exo*-Diels–Alder reaction to give (−)-solanapyrone A.[12] Prior to this report a number of Diels–Alder catalytic antibodies had been generated and these were discussed in detail in the previous review.[1] Detailed mechanistic and structural studies have been conducted on three of these antibodies and these are discussed in section 3.6. For comparison RNA aptamers with Diels–Alderase activity have also been reported as described in section 7.4.

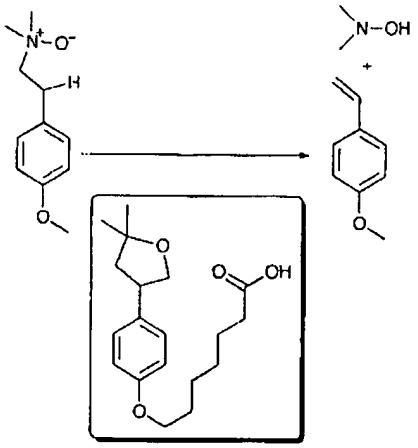
More recently Janda and co-workers have reported the preparation of antibodies capable of catalysing the 1,3-dipolar cycloaddition of benzonitrile *N*-oxide and *N*,*N*-dimethylacrylamide (Scheme 8).[13] In this case a more flexible hapten structure based on a benzyl carbamate core was used to elicit antibodies capable of catalysing the [3+2] pericyclic process. The best catalyst of the two identified, 29G12, catalysed the formation of the favoured 5-(*R*)-acylisoxazoline in 98% ee. This demonstrates the exquisite regio- and stereoselectivity that can be imparted by an antibody catalyst, even one generated using an achiral hapten. Further evidence of this is presented in section 2.11. A comparison of the activation parameters for the reaction reveals that antibody 29G12 operates by a significant reduction in the activation enthalpy for the reaction rather than, as might be expected, the activation entropy. This phenomenon has been seen previously with the Diels–Alderase antibody 1E9 and indicates that we currently have a poor understanding of the factors effecting catalysis in these pericyclic systems.

2.5 [2,3]-Sigmatropic rearrangements

Antibody catalysed [3,3]-sigmatropic rearrangements such as the Claisen and Cope rearrangements have both been reported previously.[1] These depend on the antibody binding site functioning as both an entropy trap, pre-organising the substrate into a conformation close to that of the reaction transition-state, and as a low-dielectric environment (*cf.* water) which is more amenable to the charge dispersion that occurs in moving from ground to transition-state. In an extension to these studies, Schultz and Braisted turned their attention to the Cope

† The IUPAC name for 2-norbornyl is bicyclo[2.2.1]heptan-2-yl.

elimination of an *N*-oxide, a [2,3]-sigmatropic rearrangement with a five-membered pericyclic transition-state.[14] In order to generate a suitable hydrophobic binding pocket they used a substituted furan hapten and found that one of the twenty-three antibodies obtained was able to catalyse the Cope elimination of the substrate shown (Scheme 9), but not of a demethylated version, or a related sulfoxide. The rate acceleration of 10^3 for antibody 21B12.1 closely correlates with the K_M/K_i ratio, indicating that the catalysis is the result of preferential binding to the transition state with both entropic and enthalpic activation terms being reduced.



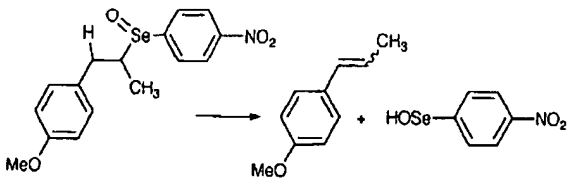
1 catalytic antibody from 23,
21B12.1: $k_{cat} = 1.44 \times 10^{-3}$ h^{-1}, $K_M = 235$ μmol dm^{-3},
$k_{cat}/k_{uncat} = 910$, $K_M/K_i = 1.2$

Scheme 9

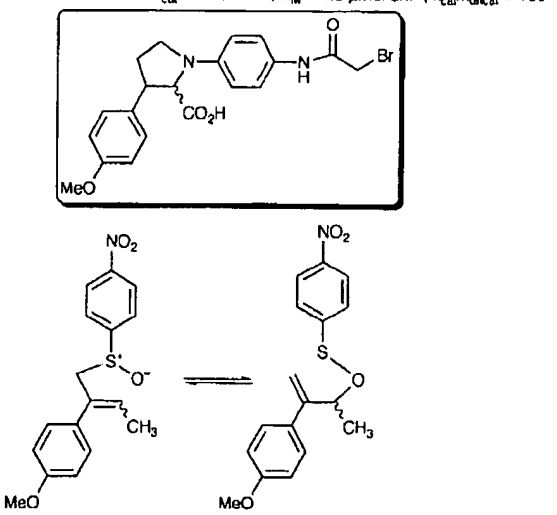
More recently Hilvert and co-workers have reported the antibody catalysed *syn*-elimination of a selenoxide,[15] and the related allylic sulfoxide-sulfenate (Evans–Mislow) rearrangements[16] (Scheme 10) which both share a five-membered pericyclic transition-state. Haptens based on substituted prolines were used to elicit twenty eight monoclonal antibodies against the *cis*-hapten, and twenty against the *trans*-hapten. From these, three antibodies capable of catalysing the selenoxide elimination were identified (SZ-*cis*-39C11, SZ-*cis*-42F7, SZ-*trans*-28F8). The *cis*-hapten appears to have elicited enantioselective antibodies whilst the antibody elicited against the *trans*-hapten can accommodate both enantiomers. As with the Kemp elimination above, bovine serum albumin is also capable of catalysing the selenoxide elimination of these substrates ($k_{cat} = 0.0022$ min^{-1}, $K_M = 170$ μmol dm^{-3}), but it does not discriminate between substrates, or exhibit any product selectivity.[16]

2.6 Ester hydrolysis

The first examples of catalytic antibodies reported exhibited esterase activity.[1] This is still an area of interest with groups attempting to develop sequence-specific protease-like antibodies. The early haptens used to elicit hydrolytic antibodies were mainly based on a tetrahedral phosphorus(v) core, chosen to mimic the tetrahedral transition-states on the ester/amide hydrolysis pathways. Whilst much success has been achieved with these systems, this has mainly been with activated ester or amide substrates. The ultimate goal of efficient unactivated peptide bond cleavage has yet to be attained. A number of alternative strategies besides transition state mimicry have also been tested. Tantillo and Houk have conducted a detailed *ab initio* study of the addition–elimination (hydrolysis) reaction of phenyl acetate and hydroxide ion, and have concluded that aryl phosphonates while being reasonable geometric mimics of the rate-determining addition transition-state are significantly poorer from an electrostatic perspective.[17] Simultaneously



3 from 48
SZ-*cis* 39C11: $k_{cat} = 0.039$ min^{-1}, $K_M = 13$ μmol dm^{-3}, $k_{cat}/k_{uncat} = 36$
SZ-*cis*-42F7: $k_{cat} = 0.067$ min^{-1}, $K_M = 4$ μmol dm^{-3}, $k_{cat}/k_{uncat} = 62$
SZ-*trans*-28F8: $k_{cat} = 0.18$ min^{-1}, $K_M = 1.5$ μmol dm^{-3}, $k_{cat}/k_{uncat} = 160$



3 from 48
SZ-*cis*-39C11: $k_{cat} = 0.14$ min^{-1}, $K_M = 28$ μmol dm^{-3},
$k_{cat}/k_{uncat} = 820$ (*cis* substrate)
SZ-*trans*-28F8: $k_{cat} = 0.090$ min^{-1}, $K_M = 3.3$ μmol dm^{-3},
$k_{cat}/k_{uncat} = 160$ (*trans* substrate)
BSA: $k_{cat} = 0.022$ min^{-1}, $K_M = 470$ μmol dm^{-3}, $k_{cat}/k_{uncat} = 130$ (*cis* substrate)
$k_{cat} = 0.10$ min^{-1}, $K_M = 230$ μmol dm^{-3}, $k_{cat}/k_{uncat} = 170$ (*trans* substrate)

Scheme 10

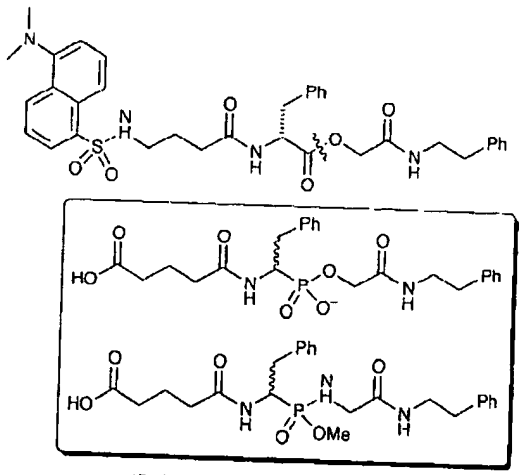
Kakinuma *et al.* compared the use of structurally similar phosphonate and neutral phosphonamidate haptens for their ability to generate antibodies in MRL/*lpr* mice (Scheme 11).[18] They found that the phosphonate elicited seventeen catalytic antibodies from forty-one monoclonals whilst the phosphonamidate hapten elicited none from twenty seven that bound the hapten. They rationalised the results in terms of the negatively charged phosphonate being able to induce groups suitable for stabilising an oxyanion that the phosphoramidate was not. However, a more detailed understanding of the mechanism(s) of action of the catalytic antibodies produced in this study is required before any firm conclusions can be reached.

Syntheses of a variety of other phosphonate containing haptens have been reported for a range of different reactions: phospholipase A_2;[19] hydrolysis of a scalemic pyrrolidine diester synthon for ptilomycalin A,[20] and hydrolysis of cocaine.[21] However, catalytic antibodies have yet to be reported that have been elicited against these haptens.

An example of hydrolytic antibodies elicited against a phosphonate hapten that have potential uses in organic synthesis is described by Fujii and co-workers.[22] Generally catalytic antibodies display an Epicurean specificity for their substrate. This may be an advantage in medical or sensor applications, but is less useful if the antibody is to be used in synthesis, where a broad, but predictable substrate specificity is preferred. Fujii and co-workers have succeeded in developing two antibodies which have both high enantioselectivity and broad substrate specificity in this case for the hydrolysis of either the *para*-nitrobenzyl esters of a range of carbobenzoxy-protected D- or L-amino acids (Scheme 12). The reaction scope of the L-selective (7G12) and D-selective (3G2) antibodies is summarised in Table 1.

Table 1 Two antibodies for enantioselective hydrolysis of *N*-Cbz-amino acid 4-nitrobenzyl esters

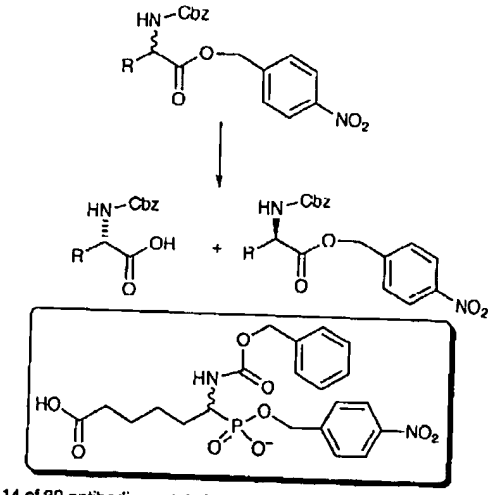
Substrate (Z-X-4-NO_2PhCH_2)	Antibody 7G12 K_M/μmol dm^{-3}	$10^2 \times k_{cat}$/min^{-1}	Substrate (Z-X-4-NO_2PhCH_2)	Antibody 3G2 K_m/μmol dm^{-3}	$10^2 \times k_{cat}$/min^{-1}
L-Ala	13	28	D-Ala	16	2.1
L-Leu	23	37	D-Leu	21	1.0
L-Val	36	28	D-Val	41	0.9
L-Phe	4.9	24	D-Phe	5.5	0.3
L-*p*-HydroxyPhe	64	120	D-*p*-HydroxyPhe	27	6.2



17 of 41 catalytic against phosphonate
0 of 27 against methylphosphonamidate

MS 6-164; k_{cat} = 0.43, K_M = 2.8 μmol dm^{-3}, k_{cat}/k_{uncat} = 4300, K_d = 0.61 nmol dm^{-3}

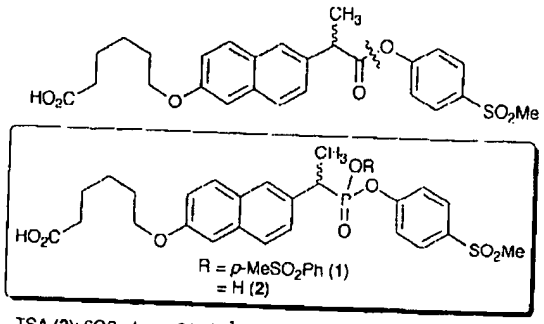
Scheme 11



14 of 39 antibodies catalytic
7G12 (L-specific): k_{cat} = 7.0 x 10^{-2} min^{-1}, K_M = 13 μmol dm^{-3}, k_{cat}/k_{uncat} = 3700
3G2 (D-specific): k_{cat} = 3.3 x 10^{-2} min^{-1}, K_M = 5.4 μmol dm^{-3}, k_{cat}/k_{uncat} = 1700

Scheme 12

Janda and co-workers have continued to explore the reactive immunisation concept described in the previous review,[1] to generate antibodies capable of catalysing the hydrolysis of (*S*)- or (*R*)-naproxen (Scheme 13).[23] In this case the bis-(*p*-methylsulfonylphenyl)phosphonate hapten is slowly hydrolysed in solution such that the immune system of the mouse is challenged by mechanism-based inhibitor and transition-state analogue structures simultaneously. The best antibody generated by this approach, 5A9, has a high turnover rate (k_{cat} = 2.3 min^{-1}), but only a moderate enantioselectivity for (*S*)-



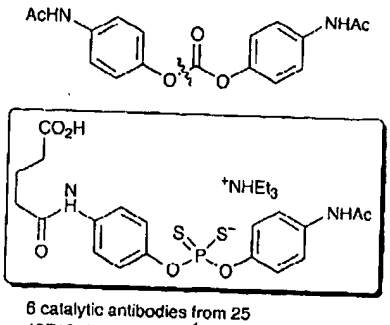
TSA (2): 6G6: k_{cat} = 81min^{-1}, K_M = 890 μmol dm^{-3}, k_{cat}/k_{uncat} = 19

RI(1+2): 15G12 : k_{cat} = 28 min^{-1}, K_M = 300 μmol dm^{-3}, k_{cat}/k_{uncat} = 6.6

Scheme 13

naproxen. Interestingly, when the immunisation was repeated using only the mono-(*p*-methylsulfonylphenyl)phosphonate, thought to resemble the transition-state, the enantioselectivity of the catalytic antibodies was improved (>98% ee for antibodies 6G6, 12C8, and 12D9). However, product inhibition was a significant problem with these antibodies. The rate enhancement (k_{cat}/k_{uncat}) correlated well for those antibodies raised using the transition-state analogue only, but underestimated the catalytic efficiency of those generated using reactive immunisation.

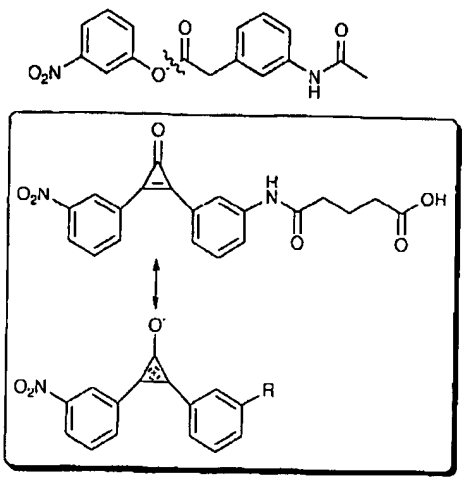
Brümmer *et al.* have examined phosphorodithioates as haptens for acyl transfer reactions (Scheme 14).[24] They reasoned that the milder polarisation of the P=S bond and the longer P–S bond lengths better mimicked the tetrahedral transition-state of acyl group transfer and hydrolysis. From the twenty-five monoclonal antibodies found to bind the hapten, six were found to catalyse the hydrolysis of a carbonate and a structurally related ester. However, other structural modifications to the substrate were not tolerated. In comparison with other carbonate hydrolysing antibodies that have been produced, the phosphorodithioate appears to elicit antibodies of similar efficency.



6 catalytic antibodies from 25
48F10: k_{cat} = 2.7 min^{-1}, k_{cat}/k_{uncat}= 3 x 10^4, K_i = 1.2 μmol dm^{-3}

Scheme 14

Grynszpan and Keinan have demonstrated that a cyclopropenone hapten is capable of eliciting antibodies capable of hydrolysing simple aryl esters (Scheme 15).[25] This planar hapten has a significant population existing in the aromatic cyclo-



5 catalytic antibodies from 15,
12G2: k_{cat} = 1.4 min^{-1}, K_M = 480 μmol dm^{-3}, k_{cat}/k_{uncat} = 200

Scheme 15

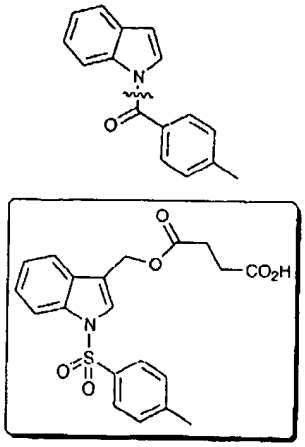
propenium form with a positive charge delocalised over the cyclopropane ring and a negative charge on the oxygen. The authors have chosen this structure to demonstrate that electrostatic factors play an equal or more significant role in the antibody catalysed hydrolysis relative to reaction geometry and shape complementarity to the tetrahedral transition-state.

2.7 Amide hydrolysis

Sequence specific peptide hydrolysis is still a major target for researchers in the catalytic antibody area for which the standard approach of using a transition-state analogue has met with very limited success, one of the few examples being the hydrolysis of *N*-*p*-toluoylindole catalysed by antibodies raised against *N*-*p*-tolylsulfonylindole as reported by Benedetti *et al.* (Scheme 16).[26] This is a rare example of the non-charged sulfonamide eliciting hydrolytic antibodies. Janda and co-workers have used an alternative approach involving a boronic acid to select a diastereoselective catalyst for the hydrolysis of a primary carboxamide of a prolyl tripeptide from a phage-displayed F_{ab} library (Scheme 17).[27] The boronic acid again has two roles. It can either act as a Lewis acid when present in its trigonal form (pK_a ~8.0) selecting antibody binding sites with nucleophilic residues, or in the tetrahedral hydrated form as a transition-state analogue. It was interesting to note that none of the twenty-five monoclonal antibodies produced by the hybridoma technique was found to be catalytic. Instead the catalytic Fab BL25 was isolated from a phage display library of 2×10^8 members which had been produced from an immunised mouse.

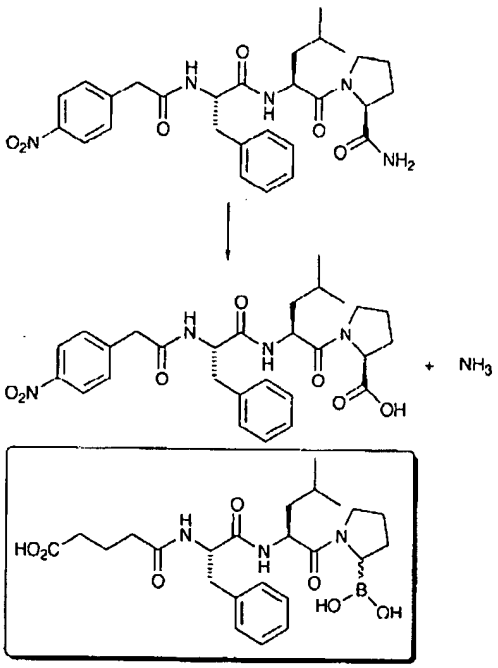
Antibodies with β-lactamase activity have also been isolated from a library of 4.8×10^{10} phage particles generated against a penam sulfone. Penam sulfones are known to be potent mechanism based inhibitors of β-lactamase enzymes, forming stable acyl-enzyme intermediates. Catalytically active phage-displayed scFv's (single-chain Fragment variable) were identified by four rounds of panning against immobilised penam sulfones. Two single-chain antibody fragments, FT6 and FT12 were then characterised in detail for their ability to catalyse the hydrolysis of the β-lactam ring of ampicillin (Scheme 18).[28] As expected time-dependent inhibition of both antibody fragments was observed on incubation with the sulfone penam hapten, FT6 also catalysed the hydrolysis of the β-lactam ring of the sulfone. Antibodies FT6 and FT12 were found to be highly homologous, differing by a total of only seven residues in the complementarity determining regions (CDRs), whilst possessing identical length hypervariable loops.

The only other new amide hydrolysing antibodies are those reported by Masamune and co-workers[29] who have shown that



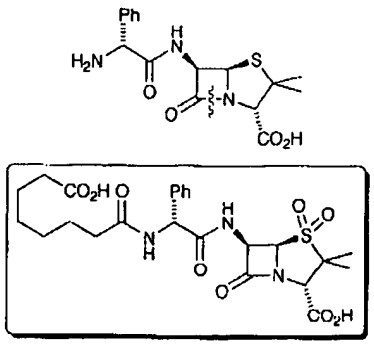
2 catalytic antibodies from 7.
312D6: $k_{cat} = 7.6 \times 10^{-6}$ s^{-1}, K_M = 38 μmol dm^{-3}, k_{cat}/k_{uncat} = 750, K_i = 1.0 μmol dm^{-3}

Scheme 16



selected from 2×10^8 phage library
BL25 Fab: k_{cat} = 0.003 min^{-1}, K_M = 150 μmol dm^{-3}, $k_{cat}/k_{uncat} = 4 \times 10^4$

Scheme 17



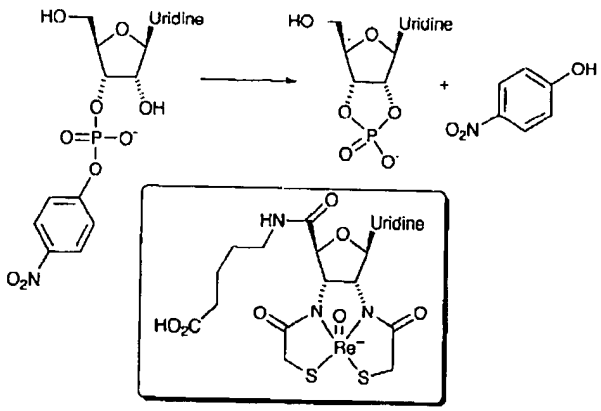
FT6 (scFv): k_{cat} = 2.9 x 10^{-1} min^{-1}, K_M = 560 μmol dm^{-3}, k_{cat}/k_{uncat} = 5200

Scheme 18

an amide can be cleaved *via* either an intra- or intermolecular N→O acyl transfer reaction. These workers found that cyclic phosphoramidate or β-hydroxy pyridinium haptens were successful in eliciting catalytic antibodies, but *N*-oxides were not.

2.8 Phosphate ester hydrolysis

The hydrolysis of the phosphate diester bond is central to living systems and a number of groups have looked at the creation of catalytic antibodies which can either mimic nucleases, or be used for the degradation of organophosphate pesticides or chemical warfare agents. Janda and co-workers have used both an oxorhenium(v) chelate transition-state analogue (Scheme 19),[30] and a 'bait & switch' approach (Scheme 20)[31] to catalyse phosphodiester bond cleavage. First, they demonstrated that a stable pentacoordinate oxorhenium compound which mimics the distorted trigonal bipyramidal/square-planar transition state was capable of raising antibodies which catalysed the cleavage of uridine 3'-(*p*-nitrophenyl) phosphate. Alternatively by incorporating a dimethylamino group at the 2 position of the 2-deoxyribose, an active site base (histidine?) could be induced which would activate the 2-hydroxy to attack the phosphodiester bond. Antibody MATT.F-1 which catalyses the cyclisation and cleavage of a 3-(*p*-nitrophenylphospho)-1-deoxyribitol was isolated from this immunisation. A comparison of the two approaches indicates that the antibodies raised against the 'bait & switch' hapten are the more efficient catalysts. An alternative hapten incorporating a P(O)–O–N bond appears not to have been successful in the generation of phosphodiesterase antibodies.[32]

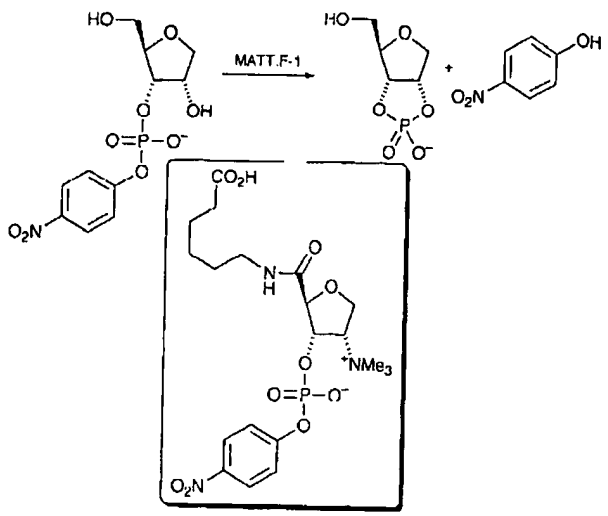


3 catalytic antibodies from 25,
2G12: $k_{cat} = 1.53 \times 10^{-3}$ s^{-1}, $K_M = 240$ μmol dm^{-3},
$k_{cat}/k_{uncat} = 312$, $K_M/K_I = 600$

Scheme 19

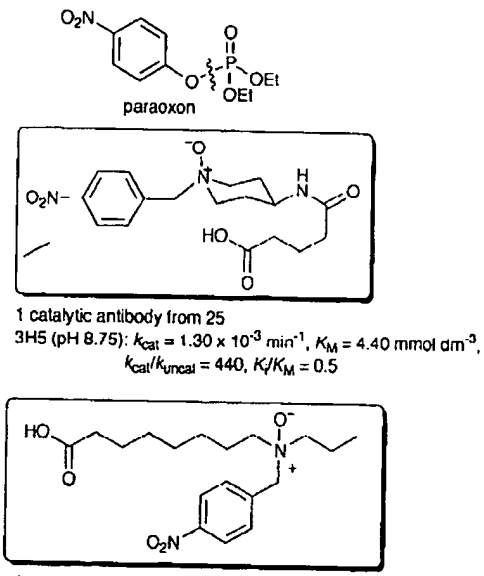
Several groups have been involved in the development of antibodies capable of hydrolysing the fluorophosphate diesters found in the G-series nerve agents sarin, soman and the phosphotriesters found in the insecticides paraoxon, parathion, and diazinon. A number of new antibody catalysts have been identified since 1995. Janda and co-workers have demonstrated that both flexible and constrained amine oxides are capable of eliciting antibodies that will catalyse the hydrolysis of paraoxon and related phosphotriesters (Scheme 21),[27] whilst Yli-Kauhaluoma *et al.* have demonstrated that the pentacoordinate methyloxyphosphorane originally prepared by Brimfield *et al.*[33] could be used to generate antibodies capable of hydrolysing the nerve agent soman.[34]

A number of pentacoordinate phosphorus and vanadium compounds have been reported for use as haptens to elicit phosphodiester hydrolysing antibodies,[35] but it remains to be seen if these are successful.



$k_{cat} = 0.44$ min^{-1}, $K_M = 104$ μmol dm^{-3},
$k_{cat}/k_{uncat} = 1.65 \times 10^3$, $K_I/K_M = 2.1$

Scheme 20



1 catalytic antibody from 25
3H5 (pH 8.75): $k_{cat} = 1.30 \times 10^{-3}$ min^{-1}, $K_M = 4.40$ mmol dm^{-3},
$k_{cat}/k_{uncat} = 440$, $K_I/K_M = 0.5$

7catalytic antibodies from 20
1H9(pH 8.77): $k_{cat} = 3.73 \times 10^{-4}$ min^{-1}, $K_M = 1.12$ mmol dm^{-3},
$k_{cat}/k_{uncat} = 56$, $K_I/K_M = 2.1$

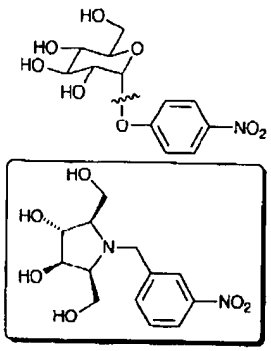
Scheme 21

2.9 Glycoside hydrolysis

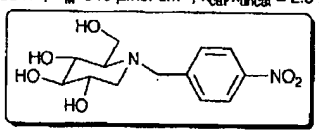
Reymond and co-workers demonstrated that their ubiquitous *N*-alkyl-*N*-methyl-3-(glutarylamidomethyl)piperidinium hapten is capable of eliciting antibodies which are capable of hydrolysing a simple ketal demonstrating what they term 'primordial glycosidase' activity.[36] More recently Youn and co-workers have used a variety of immunisation techniques (*in vitro* immunisation, SJL/J auto-immune prone mice, and conventional Balb/c mice) to generate antibodies capable of hydrolysing *p*-nitro-α-D-glucopyranoside using either a five-membered iminocyclitol, or a *N*-*p*-nitrobenzyl 1-deoxynojirimycin hapten (Scheme 22).[37] Each of the immunisation techniques generated >50 primary clones of which twenty clones in each case were studied further. The frequency of catalytic clones is summarised in Table 2. This demonstrates that the autoimmune mice, or *in vitro* immunisation produced a much greater number of catalysts suggesting that the intact immune system of a healthy animal may have a mechanism of down regulating catalytic antibody secreting B-cells. One of the catalytic antibodies generated by the *in vitro*

Table 2 Occurrence of catalytic clones with α-glycosidase activity using different immunisation methods

Immunisation method	Clones assayed	Clones with catalytic activity	Frequency (%)
Balb/c mice immunisation	17	1	6
SJL/J immunisation	15	14	93
In vitro immunisation	14	11	79



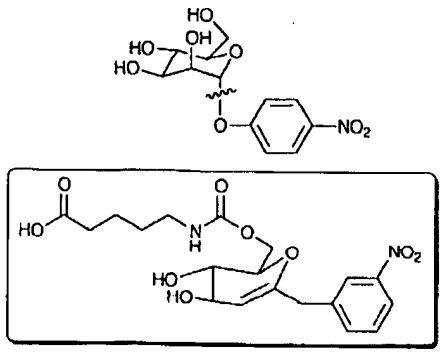
2 catalytic antibodies from 14 by *in vitro* immunisation
Ab24 (*p*-nitrophenyl-β-D-glucopyranoside):
k_{cat} = 0.02 h^{-1}, K_M=160 μmol dm^{-3}, k_{cat}/k_{uncat} = 2.2 x 10^4, K_i = 380 μmol dm^{-3}
Ab21 (*p*-nitrophenyl-β-D-galactopyranoside):
k_{cat} = 0.035 h^{-1}, K_M=310 μmol dm^{-3}, k_{cat}/k_{uncat} = 2.5 x 10^4, K_i = 60 μmol dm^{-3}



13 catalytic antibodies from 20 by *in vitro* immunisation
Ab 4f4f (*p*-nitrophenyl-β-D-glucopyranoside):
k_{cat} = 2.8 x 10^{-3} min^{-1}, K_M=22 μmol dm^{-3}, k_{cat}/k_{uncat} = 2.0 x 10^5

Scheme 22

immunisation method, 4f4f, was characterised in more detail.[38] The pH–rate profile data and chemical modification studies suggest that an active-site Asp or Glu residue is functioning as a general acid in this antibody. All of the antibodies produced in this study hydrolysed only the β-glycoside anomer. Yu has reported that a glucal hapten could be used to generate an antibody (405.4), capable of hydrolysing α-mannosides (Scheme 23).[39] It is not clear if these antibodies were raised by *in vivo* or *in vitro* methods. These catalysts were inactive against the β-anomer, and again chemical modification implicates one or more active-site carboxylates in the reaction mechanism. More recently antibody 4f4f has been cloned and expressed as a single-chain F_V fragment.[40]

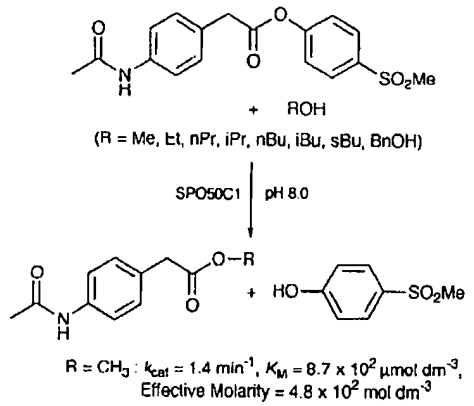


5 catalytic antibodies from 60 by *in vivo* immunisation
405.4 (*p*-nitrophenyl-β-D-glucopyranoside):
k_{cat} = 0.19 d^{-1}, K_M= 1.0 mmol dm^{-3},
k_{cat}/k_{uncat} = 1.1 x 10^5, K_i = 220 μmol dm^{-3}

Scheme 23

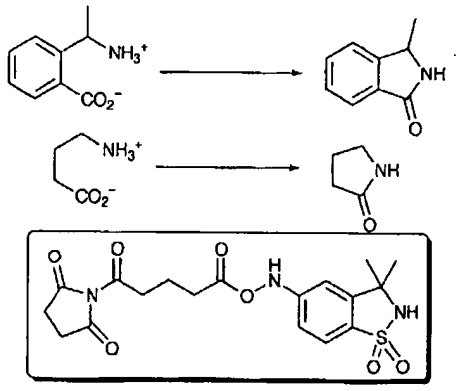
2.10 Ester and amide bond formation in water

Janda and co-workers have demonstrated that antibody SPO50C1 (Scheme 24) produced using the 'reactive immunisation' strategy was capable of catalysing the formation of esters in water.[41] Antibody SPO50C1 was found to catalyse the transesterification of a broad range of primary and secondary alkyl alcohols. The detection of a stoichiometric 'burst' in the formation of phenol in the hydrolytic mechanism of this antibody when studied under pre-steady-state conditions suggested the formation of an acyl enzyme intermediate in the mechanism. The hydrophobic nature of the antigen-binding site appears to favour the trapping of this intermediate with an alkyl alcohol rather than water.



Scheme 24

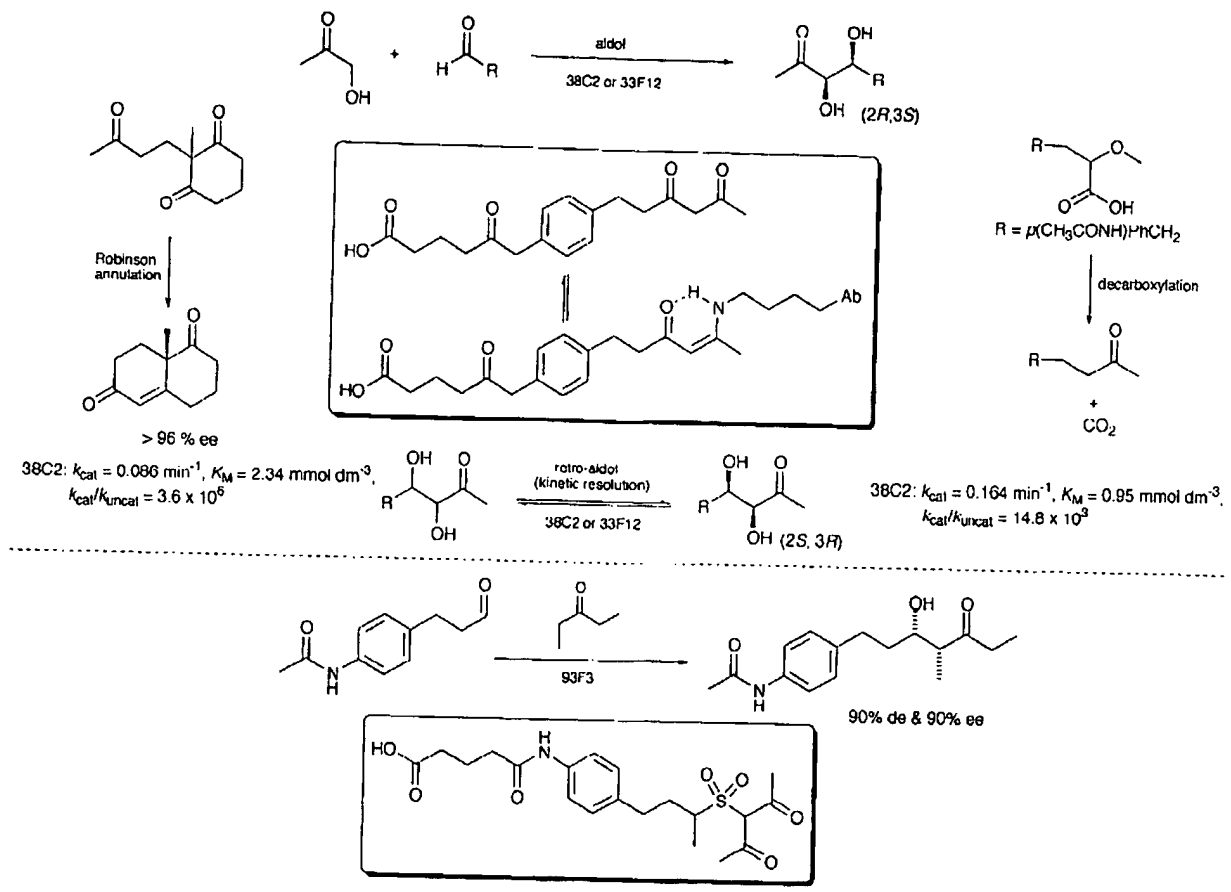
Further mechanistic studies have been conducted on the antibody 'ligase' 16G3 previously reported by Benkovic, Hirschmann and co-workers which is capable of catalysing the cyclisation of D-Trp-Gly-Pal-Pro-Gly-Phe-*p*-nitrophenyl ester (Pal = L-3-pyridylalanine) with a 22-fold rate enhancement.[42] Zherdev and co-workers have reported the use of a cyclic-sulfonamide to catalyse the lactamisation of both 2-aminomethylbenzoate and GABA (Scheme 25).[43] Kinetic parameters have yet to be reported on these antibodies.



Scheme 25

2.11 Aldol and retro-aldol reactions

Reactive immunisation was used in 1995 to generate two of the most synthetically versatile antibody catalysts.[44] A β-diketone containing hapten was designed to stimulate the production of antibodies containing a nucleophilic lysine in their antigen binding site. It was proposed that this would be capable of forming Schiff-bases with suitable carbonyl compounds and these could then undergo aldol or retro-aldol reactions in a manner analogous to the mechanism of action of natural class I aldolases (Scheme 26). The chiral environment of the antigen-binding pocket would be expected to lead to some stereo-



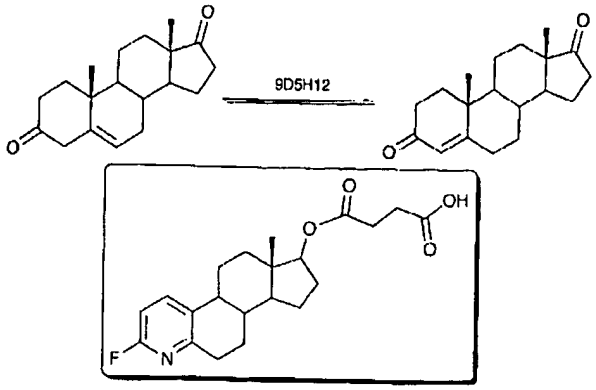
Scheme 26

selectivity in the antigen binding pocket. Using this approach two efficient antibody catalysts 33F12 and 38C2 were identified by monitoring the formation of a vinylogous amide (conjugated enamine) product (λ_{max} = 316 nm; $\varepsilon \sim 15000$ cm^{-1} dm^3 mol^{-1}) upon mixing with acetylacetone. The two antibodies were found to catalyse the diastereoselective addition of ketones to the *Re*-face of the enamine to give the (4*S*,5*S*)-aldol in >96% de. Having demonstrated that intermolecular aldol reactions could be catalysed by these antibodies, the researchers then investigated their ability to catalyse an intramolecular aldol condensation–dehydration (Robinson annulation), leading to the enantioselective preparation of the (*S*)-Wieland–Miescher ketone, a key intermediate in many natural product syntheses from an achiral triketone in >95% ee (see sections 6.1 and 6.2). Recently, a modified form of the diketone hapten incorporating a sulfone group to mimic the tetrahedral geometry generated at the carbonyl acceptor centre during the reaction has been used to produce catalytic antibodies with complementary enantio- and diastereoselectivity to 38C3/33F12.[45] Nine out of seventeen monoclonal haptens were found to be catalytic with antibodies 93F3 and 84G3 being both efficient catalysts and having antipodal activity to 38C3/33F12. These new antibodies have yet to be fully exploited in synthesis.

It has been demonstrated that the aldolase antibodies 38C2/33F12 have a much broader substrate specificity than their natural counterparts such as fructose 1,6-diphosphate aldolase. They are capable of catalysing aldehyde–aldehyde, aldehyde–ketone and ketone–ketone condensations.[46] The antibodies are capable of converting both aliphatic open chain and cyclic ketones into donor enamines, and also catalysing the decarboxylation of β-keto acids.[47] The Fab of antibody 33F12 has been crystallised and its structure solved (Section 3.9).[48] The key lysine residue has been identified (Lys^{H93}), and this sits at the bottom of a 13.5 Å cleft formed by a large number of mainly hydrophobic amino acids that are conserved in the case of antibody 38C2. These antibodies have also been tested for their ability to catalyse the isomerisation of a Δ^5-3 ketosteroid to a Δ^4-3-ketosteroid (Scheme 27), again through the involvement of a Schiff base intermediate. The bacterial ketosteroid isomerase uses the hydroxy of tyrosine-14 to activate the carbonyl group through hydrogen bonding, and then the carboxylate of aspartic acid-38 to act as a base to generate the enol intermediate. Marquet and co-workers have previously reported using 3-fluoro-4-azacstradiol-17-hemisuccinate to generate an antibody which could isomerase androst-5-ene-3,17-dione (Scheme 27).[49] The mechanism of action of this antibody is currently unclear; however, the hapten may have elicited a suitably positioned carboxylate to complement the positively charged pyridinium centre. These two examples offer a comparison of the use of bait and switch and reactive-immunisation methods, respectively, for generating antibody catalysts.

Antibody 38C2 has recently been commercialised (Aldrich cat. no. 47,995-0). Its synthetic utility has been demonstrated in the preparation of key intermediates in the synthesis of brevicomins,[50] 1-deoxy-L-xylulose,[51] and the retro-aldol condensation in the resolution of β-hydroxyketones[52] including tertiary aldols (section 6.2).[53] The addition of palladium(II) species to aldol reactions involving antibodies 38C2 and 33F12 was found to improve the reaction rate and enantioselectivity of the condensation with certain substrates.[54] The authors suggest that the metal ions are binding not at the active-site, but at an allosteric site which promotes the active site to form a cavity which better binds the aldol transition-state (remembering that the hapten was not a transition state analogue (TSA) in this case and hence the molecular recognition to the TS would be sub-optimal). The antibody has also been shown to form a Schiff base with retinal so mimicking the visual protein opsin.[55]

In order to produce aldolase antibodies with improved kinetic properties and different regio- and stereoselectivities Lerner and Barbas redesigned the β-diketone hapten to

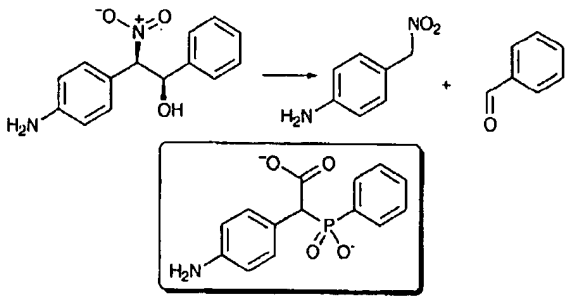


1 catalytic antibody from 5
9D5H12: k_{cat} = 2.25 min^{-1}, K_i = 160 μmol dm^{-3}.
k_{cat}/k_{uncat} = 830, K_i = 130 μmol dm^{-3}
Via Schiff base (aldolase antibody)
38C2: k_{cat} = 0034 min^{-1}, k_{cat}/k_{uncat} = 15

Scheme 27

incorporate a sulfone to mimic the tetrahedral geometry formed at the electrophilic carbonyl centre post carbon–carbon bond formation.[56] Nine out of seventeen monoclonal antibodies elicited against this hapten were found to be catalytically active, a significant improvement on the previous hapten of which two out of twenty were catalytic. More importantly antibody 93F3 was found to exhibit complementary enantio- and diastereoselectivity to antibodies 38C2/33F12. Reaction of pentan-3-one with an aldehyde acceptor in the presence of antibody 93F3 gave the *syn*-α-isomer in 90% de and 90% ee. The same reaction with antibody 38C2 gave rise to the *anti*-α-isomer in 62% de and 59% ee.

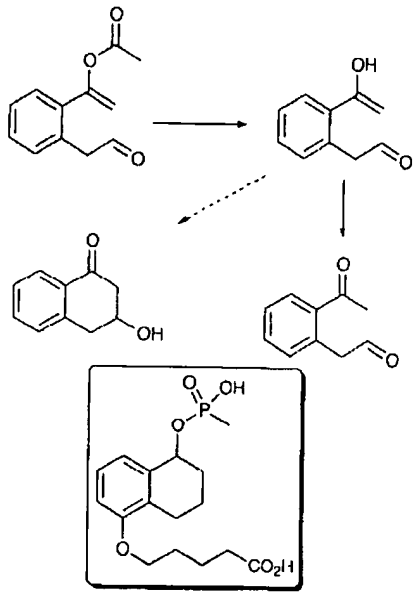
Schultz *et al.* also investigated the generation of an aldolase antibody capable of catalysing the condensation of benzaldehyde and phenylacetone.[57] They used a phosphinate hapten in order to mimic the aldol transition state, but found that none of the twenty monoclonal antibodies that bound the transition state analogue promoted this reaction. Screening of alternative substrates revealed that one of the antibodies (29C5.1) was capable of catalysing a retro-(aldol) Henry reaction on the phenylnitromethane–benzaldehyde aldol product with a 2:1 preference for the *syn* diastereoisomer over the *anti* (Scheme 28). Chemical modification studies of 29C5.1 with diethyl pyrocarbonate are consistent with a binding site histidine.



1 catalytic antibody from 20
29C5.1: k_{cat}/K_M = 125 mol dm^{-3} min^{-1}, K_i = 2.6 μmol dm^{-3}
Syn isomer preferentially degraded 2:1

Scheme 28

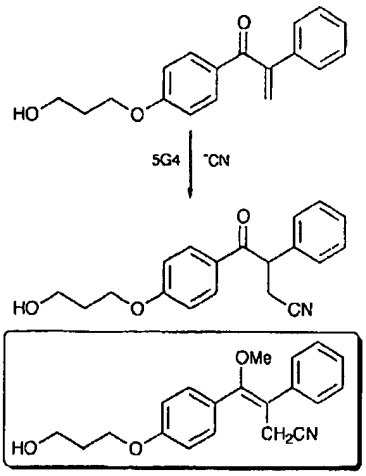
Mu and Gibbs have reported an alternative strategy for generating aldolase antibodies using antibody-catalysed enol ester hydrolysis to trigger aldolisation (Scheme 29).[58] However, after finding difficulties with the synthesis of their proposed hapten, it was found that polyclonal antibodies generated against a compromise structure were only capable of hydrolysis and not of the desired aldol cyclisation.



Scheme 29

2.12 Michael addition

Cook *et al.* have produced 4 antibodies which catalyse the addition of cyanide to an α,β-unsaturated ketone (Scheme 30).[59] They employed an enol ether hapten, reasoning that having a planar (sp^2) β-carbon in the hapten would minimise product inhibition, as this carbon is tetrahedral in the nitrile product. A similar reaction (although not a C–C bond formation) is catalysed by the thiol-S-transferase antibodies reported by Schultz and co-workers (Scheme 31).[60] In this case the antibody accelerates the addition of sodium 2-mercaptoethanesulfonate to both 4-nitrostyrene and 4-nitrocinnamyl alcohol, and hence is a mimic of the glutathione-S-transferase (GST) detoxification system. In this case the antibodies are found to be around only an order of magnitude lower in efficiency than GST.

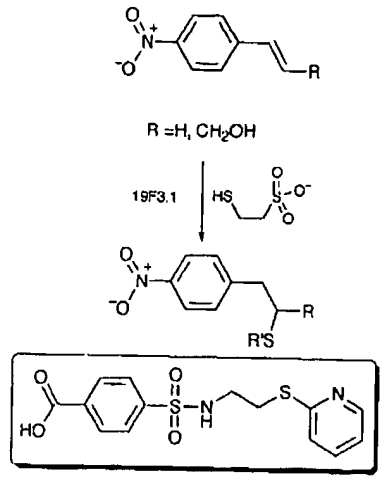


4 catalytic antibodies from 28
k_{cat} = 1.28 h^{-1}, K_{enone} = 64.3 μmol dm^{-3}, K_{CN} = 141 μmol dm^{-3},
k_{cat}/k_{uncat} = 0.03 μmol dm^{-3}, K_i = 11.2 μmol dm^{-3}

Scheme 30

2.13 Cyclisation reactions

Lerner and co-workers have continued the development of antibodies capable of controlling and catalysing cyclisation reactions with the generation of antibodies able to produce terpenoid-like products. A cyclic amidine has been used to



2 catalytic antibodies from 13
k_{cat} = 2.2 x 10^{-3} s^{-1}, K_{alkene} = 12.6 mmol dm^{-3}, K_{CN} = 141 μmol dm^{-3}, k_{cat}/k_{uncat} = 1.8 x 10^3 (pseudo 1st order), K_d = 4.2 μmol dm^{-3}

Scheme 31

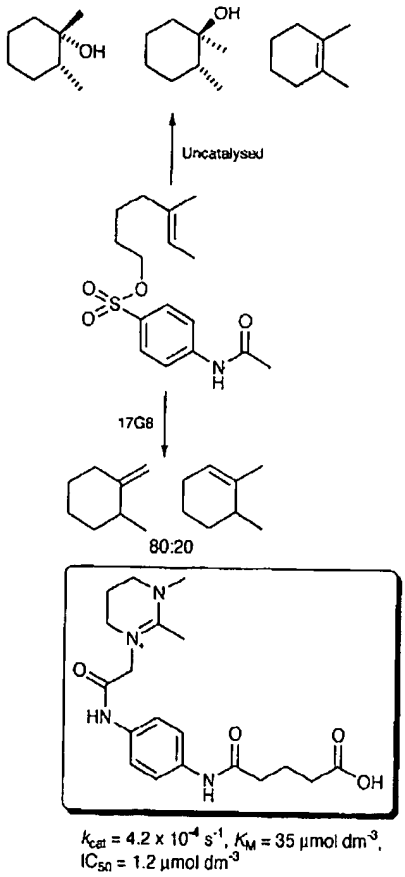
generate antibodies capable of cyclising alkene arylsulfonates to monoterpene-like products (Scheme 32),[61] which were different from the alkene and alcohols produced in the uncatalysed reaction. Using a decahydroquinoline containing an *N*-oxide, the same group were able to facilitate the formation of 3 closely related bridge-methylated decalins from a diene precursor using antibody HA5-19A4 (Scheme 33).[62] The Fab from this antibody has recently been crystallised and a detailed comparison of the structure of this antibody and the terpene cyclase pentalene synthase has been presented by Christianson and co-workers.[63] It is apparent from the X-ray crystal structure that the binding site of the antibody is contoured to bind the substrate in a chair–chair conformation and that the *N*-oxide has successfully functioned in a 'bait-and-switch' strategy as discussed in section 3.8.

Most recently Hasserodt *et al.* have used a 4-aza-steroid *N*-oxide to generate three antibodies capable of catalysing the formation of the A ring of the lanosterol skeleton as well as cyclise an oxidosqualene derivative (Scheme 34).[64]

2.14 Cofactor assisted catalysts

Researchers have continued to investigate ways of adding to the repertoire of functionality available to catalytic antibodies by incorporating coenzymes and cofactors into their binding sites. Malthouse and co-workers have produced an antibody, 15A9, capable of catalysing the exchange of the α-hydrogens of glycine once it had formed a Schiff's base with pyridoxal 5'-phosphate.[65] The exchange of the *pro-S* hydrogen is accelerated an order of magnitude more than that of the *pro-R* hydrogen. The authors argue that the acceleration provided by the antibody is due to the trapping of the Schiff base in a position which allows maximal overlap between the α-C–H bond being cleaved and the π-orbitals of the pyridinium electron sink, rather than the involvement of an active-site base. Fujii and co-workers have reported the generation of an antibody (10H2) that catalyses the pyridoxal 5'-phosphate mediated aldol and retro-aldol condensation reactions of glycine and 4-acetamidobenzaldehyde (Scheme 35).[66] To prevent unwanted formation of Schiff's bases between antibody lysines and pyridoxal 5'-phosphate the antibody was pretreated with acetyl *N*-hydroxysuccinimide to modify the lysine ε-amino groups. The antibody was found to display a moderate *threo*-stereoselectivity in the retro-aldol reaction that is the opposite of that found in the uncatalysed reaction.

Barbas and co-workers have reported the use of the 'reactive immunisation' approach to identify antibodies with active site



k_{cat} = 4.2 x 10^{-4} s^{-1}, K_M = 35 μmol dm^{-3}, IC_{50} = 1.2 μmol dm^{-3}

Scheme 32

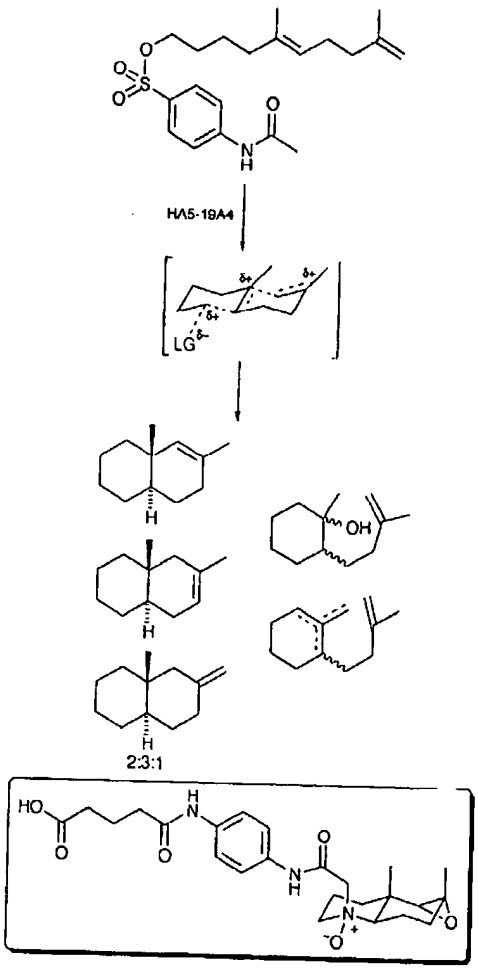
lysine nucleophiles using a 1,3-diketone hapten as described in section 2.11.[67] Lysine^{H93} of antibodies 38C2 and 33F12 was found to react with β-lactams (1-acylazetidin-2-ones). Hence a variety of β-lactams with thiazolium or pyridinium moieties appended were reacted with these two antibodies in order to position these coenzymes at the antibody binding site (Scheme 36). One of these conjugates with a thiazolium modification was found to catalyse the decarboxylation of phenylpyruvate.

2.15 Redox reactions

Research has continued into the development of a variety of porphyrin dependent catalytic antibodies. Both Schultz's and Kawamura-Konishi's groups have identified antibodies which catalyse porphyrin metal insertion reactions.[9,68] Mahy and co-workers have conducted an in-depth study into the development of peroxidase-like hemoantibodies.[69] They have demonstrated that an antibody elicited against iron(III)-α,α,α,β-*meso*-tetrakis(*o*-carboxyphenyl)porphyrin (ToCPP) was capable of catalysing the oxidation of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) with hydrogen peroxide. In this case the reaction was 5-fold faster than that with iron(III)(ToCPP) alone. Ding *et al.* have conducted a detailed kinetic study on the seleno-4A4 semi-synthetic antibody with glutathione peroxidase-like activity[70] that was described in the previous review.[1] The antibody appears to mimic the enzyme in operating by a ping pong mechanism.

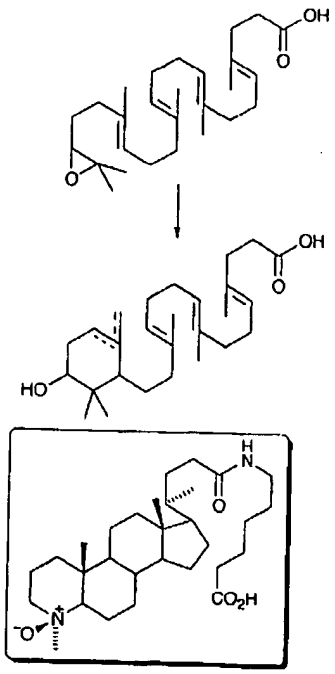
Lerner and co-workers have reported the design and synthesis of transition-state analogues for a nicotinamide mediated hydride transfer reaction.[71] To date, however, no catalytic antibodies have been reported using this hapten.

Taran and co-workers have developed an antibody capable of catalysing the decarboxylative oxidation of vanillylmandelic acid to give vanillin using the cheap inorganic oxidant sodium metaperiodate (Scheme 37).[72] Whilst this antibody is not



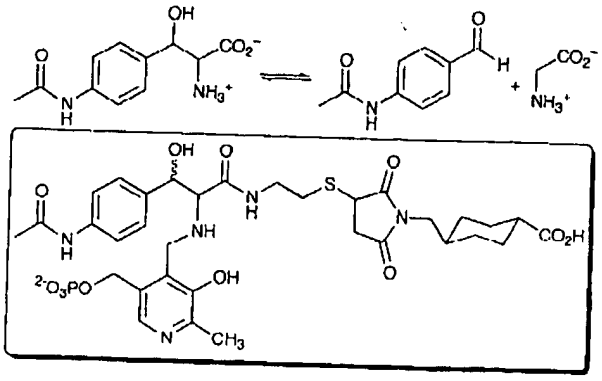
k_{cat} = 0.021 min^{-1}, K_M = 320 μmol dm^{-3},
k_{cat}/k_{uncat} = 2.3 x 10^3, K_i = 1.2 μmol dm^{-3}
(values for sulfonic acid release)

Scheme 33

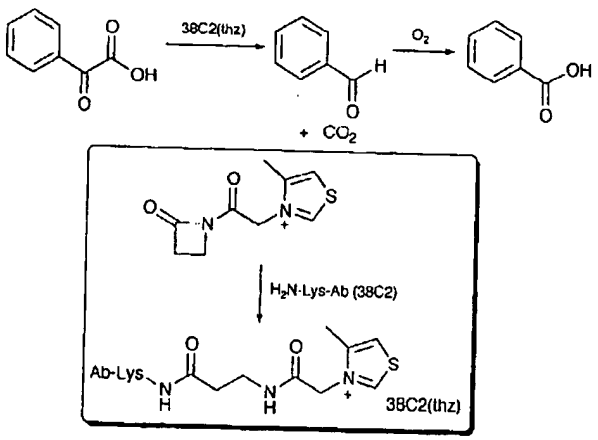


3 catalytic antibodies from 25
HA8-25A10: k_{cat} = 0.017 min^{-1}, K_M = 387 μmol dm^{-3}

Scheme 34

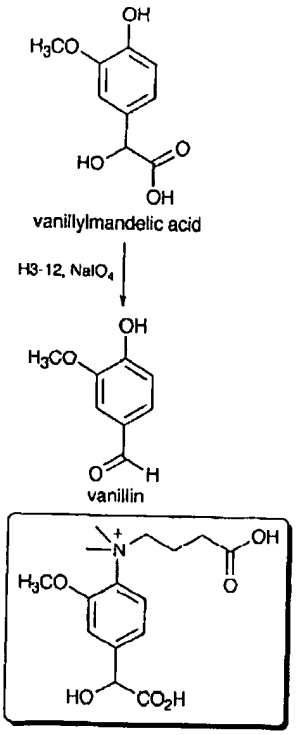


Scheme 35



38C2(thz): k_{cat}= 2.1 x 10^{-3} min^{-1}, K_M = 5.8 x 10^2 μmol dm^{-3},
k_{cat}/k_{thz} = 0.21 mol dm^{-3}

Scheme 36



1 catalytic antibody from 11
k_{cat} = 2.70 min^{-1}, K_M (vanillylmandelic acid) = 260 μmol dm^{-3}

Scheme 37

efficient enough to be used industrially, it does preferentially catalyse the oxidation of *p*-vanillylmandelic acid to give vanillin, rather than the *ortho*-isomer or other related mandelic acids.

2.16 Modification of non-catalytic antibodies into catalytic ones

Lee and co-workers have demonstrated that the monoclonal antibody specific for poly(rI), Jel 103 can be converted into a catalytically active antibody, by mutating Arg^{H96} which is positioned close to the 2'-OH of the ribose ring into a histidine.[73] The latter is capable of functioning as a base to deprotonate the 2'-OH. The catalytic efficiency (k_{cat}/K_M) of the His^{H96} mutant is 100 $dm^3\ mol^{-1}\ sec^{-1}$. This compares with a value of 10^6 $dm^3\ mol^{-1}\ sec^{-1}$ for ribonuclease T_1. In a second example,[74] the same group used site-directed mutagenesis to convert antibody Jel 42, which binds the bacterial protein HPr, into a hydrolytic antibody which cleaves the same protein. A quadruple mutant (EKHG) was found which catalysed the cleavage of the HPr protein, whilst leaving BSA untouched. At this stage it is unclear if a single amide bond is cleaved specifically.

2.17 Anti-idiotypic catalytic antibodies

A number of new catalytic antibodies have been reported, which have been produced against antibodies that are proposed to be 'internal images' of enzyme active sites. Friboulet and co-workers in Compiegne pioneered this approach with the generation of an acetylcholinesterase like catalytic antibody, and they have recently described a monoclonal antibody anti-idiotype with β-lactamase activity.[75]

Jin and co-workers generated antibody IIF_9D_8, an anti-idiotype of anti-carboxypeptidase A antibody $ID_{11}D_7$.[76] Anti-idiotype IIF_9D_8 is reported to catalyse the hydrolysis of hippuryl-DL-phenyllactic acid with $k_{cat} = 0.598\ min^{-1}$, $K_M = 0.036$ M and $k_{cat}/k_{uncat} = 30\,500$.

2.18 Polyclonal catalytic antibodies

Few new reports of polyclonal catalytic antibodies have appeared since the last review.[1] One reason for this has been the inability to separate the catalytically active antibody fraction from the non-catalytic, although strategies such as the use of hapten affinity column chromatography have been extensively investigated. The testing of hapten design by production of polyclonal antibodies is valid as a method of surveying the total immune response to a given structure, and also provides information for the possible use of catalytic antibodies *in vivo* for therapeutic purposes as described below.

Iverson and co-workers have surveyed the reproducibility of the immune response by examining the polyclonal antibodies generated against a triarylphosphonium hapten in five rabbits.[77] The rabbits all produced antibodies with broadly similar k_{on} and k_{off} rates and, with the exception of antibodies from one rabbit, all had similar catalytic activities. The rate acceleration was also found to be similar to that found in a mouse immunised with the same hapten. These results give a good indication that the response to a given hapten is reproducible and far less heterogeneous than previously thought. These researchers also found that polyaromatic haptens of increasing hydrophobicity were less successful in eliciting catalysts as they focused most of the antibody binding energy to the hydrophobic portions of the hapten and substrate rather than the transition-state mimic components.[78] In a later study of twelve phosphate and phosphonate based haptens with closely related structures, it was interesting to find that three of the enantiomeric pairings did not generate catalytic antibodies in rabbits or mice despite generating high affinity immune responses as estimated by competitive ELISA.[79] The primary reason for this appears to be the presence of benzyloxy- rather than phenolate leaving groups. These are less good leaving groups, but not sufficiently poor to change the rate-determining step from water/hydroxy attack to collapse of the tetrahedral intermediate resulting in expulsion of the leaving group (as judged by ^{18}O experiments). The lack of catalysis could be due to a combination of a more balanced mechanism with both transition-states becoming partially rate determining, and/or the increased flexibility of the benzyloxy group eliciting antibody binding pockets with lower resemblance to the rate-determining transition state trajectory.

Hu *et al.* have reported a polyclonal Diels–Alderase catalytic antibody preparation capable of catalysing the *endo*-cycloaddition of ethyl glyoxalate and a hepta-1,3-diene.[80] A bridged hapten was used, similar in concept to that previously employed by Braisted and Schultz.

Brocklehurst and co-workers have conducted an in depth study on their highly active hydrolytic sheep polyclonal antibodies prepared using a phosphate containing hapten.[81] Chemical modification and pH–rate profile studies implicate the side chains of tyrosine and arginine in the mechanism with the possibility that an aspartate or glutamate may be functioning as a general base, activating the hydrolytic water molecule.

2.19 Autoimmune catalytic antibodies

The occurrence of auto-antibodies (antibodies generated against epitopes naturally found in the host) with catalytic activity has been a controversial area since the initial report by Paul and co-workers in 1989.[82] Paul has continued to consolidate the evidence for the occurrence of catalytic auto-antibodies and establish a link with multiple myeloma or a variety of autoimmune diseases, especially thyroiditis and systemic lupus erythematosus (SLE). He has speculated that the hydrolytic antibodies fall into two types; a polyreactive fraction consisting of non-immune germline (Bence–Jones proteins), minimally mutated polyclonal IgG and light chains capable of hydrolysing a broad range of small peptides; and a more specific group of antibodies that are either vasoactive intestinal peptide (VIP) or thyroglobulin specific.[83]

In the previous review[1] several groups had reported the occurrence of RNase or DNase activity in sera from patients with SLE or hepatitis B when compared to healthy donors.[84] More recently there have also been reports of nuclease antibodies in human milk,[85] the blood and cerebrospinal fluid of patients with multiple sclerosis.[86] Sinohara *et al.* have also observed DNase activity in Bence–Jones proteins from multiple myeloma patients.[87] A detailed study of the specificity, mechanism of action and biological role of these antibodies has yet to be undertaken.

Other researchers have also reported amylolytic activity (hydrolysis of malto-oligosaccharides and *p*-nitrophenyl α-D-glucospyranoside) in antibody samples isolated from human blood and milk.[88] Again the levels of antibody-mediated catalytic activity appear to be elevated in diseased individuals (cancer, fibrinoma).

3 Mechanism of action, structure and evolution of catalytic antibodies

At the time of the last review only five catalytic antibody X-ray crystal structures had been solved whilst a model of the binding site of the amidase antibody 43C9 had been constructed based on kinetic, mutagenesis and sequence homology data.[1] As can be seen in Table 3 a much larger number of catalytic antibody structures are now available. These give us an insight into the type of binding pocket functionality that a particular hapten structure will select or induce, and hence possible mechanisms of action. Schultz's work on the identification of the hypothetical 'germline' antibody precursors given by the genes that come together in a variety of combinations to produce the naïve immune response, prior to maturation of the antibody through somatic mutation, also provides some information on

Table 3 Summary of available X-ray crystal structures for catalytic antibodies

Antibody	Reaction	PDB code	Hapten bound?	Resolution/Å	Remarks	Ref.
17E8	Ester hydrolysis	1EAP	Yes	2.50	Phosphonate	93
33F12		1A0Q	Yes	2.30		94
6D9	Ester hydrolysis	1HYX		1.80	Fab, phosphonate	95
		1HYY		1.80	Fab	95
D2.3	Ester hydrolysis	1YEC	Yes	1.90	IgG_{2A}, Fab phosphonate	96
		1YEF	Yes	2.00		97
		1YEG	*p*NO_2PhOH	2.00		97
		1YEH	No	2.55		97
						97
D2.4	Ester hydrolysis	1YED	Yes	3.10	IgG_{2A}, Fab phosphonate	96
D2.5	Ester hydrolysis	1YEE	Yes	2.20	IgG_{2A}, Fab phosphonate	96
CNJ206	Ester hydrolysis	1KNO	Yes	3.20	IgG_{2A}, Fab	98
		2GFB	No	3.00		98
48G7	Ester hydrolysis	1AJ7	Yes	2.10	Germline Fab	99
		2RCS	No	2.10	Germline Fab	99
		1HKL	No	2.70	Fab	100
		1GAF	Yes	1.95	Chimeric Fab	101
43C9	Ester/Amide hydrolysis	1M1G	Yes	N/A	F_V model	102
		43C9	No	2.20	ScF_V	103
		43CA	*p*NO_2PhOH	2.30	phosphonamidate	103
McPC603	Ester hydrolysis	1MCP	No	2.70	Fab, Anti-phosphocholine	104
		2MCP	Yes	3.10		104
7C8	Chloroamphenicol hydrolysis	1CT8	Yes	2.20	Fab	105
28B4	Oxygenation catalyst	1KEL	Yes	1.90	Fab, phosphonate	106
		1KEM	No	2.20		106
5C8	Cyclase	15C8	No	2.50	Fab	107
		25C8	Yes	2.00	Fab	107
		35C8	Yes	2.00	Fab	107
HA5-19A4	Terpene cyclase	1CF8	Yes	2.70	Fab	63
1F7	Chorismate mutase	1FIG	Yes	3.00	Fab	108
13G5	*exo*-Diels–Alder	1A3L	Yes	1.95	Fab	109
1E9	Diels–Alder	1C1E	No	1.90	Fab	113
39-A11	Diels–Alder	1A4K	Yes	2.40	Fab germline	110
		1A4J	No	2.10		110
AZ-28	Oxy-Cope	1AXS	Yes	2.60	Chimeric Fab	101
		1D5B	No	2.80	Chimeric Fab	112
		1D5I	No	2.00	Germline Fab	112
		1D6V	Yes	2.00	Germline Fab	112
33F12	Aldolase	1AXT	No	2.20	Fab	48
7G12	Chelatase	3FCT	Yes	2.20	F_v	9
Jel 103	Nuclease	1MRC	No	2.30	Non-catalytic precursor	114
		1MRD	Yes	2.40		114
		1MRE	Yes	2.30		114
		1MRF	Yes	2.40		114
Jel 42	Peptidase	2JEL	Yes	2.50	Non-catalytic precursor	74

this poorly understood phenomenon. In the case of those antibodies which bind haptens containing a phosphonate or phosphonamidate group, a common solution to binding and hence catalysis is beginning to appear which has been commented on in several more detailed reviews of this topic.[89–91] This puts into question the actual diversity of antibodies that are stimulated by an immune response from a small antigen. This appears, in practice, to be a very small fraction of the 10^7 different antibodies routinely quoted as being contained in the naïve immune response, and is consistent with data from other anti-hapten non-catalytic antibodies such as those specific for 2-phenyloxazolone.[92] The observation that only a handful of somatic mutations are required to give several orders of magnitude increase in affinity for the antigen, implies that affinity maturation also does not exploit all of conformational space, but rather rigidifies the loop conformations that are found suitable for a specific binding interaction in the initial response. Many of these mutations are of residues far from the hapten, making the possibility of rational site-specific mutagenesis mimicking somatic maturation to optimise binding for a particular hapten difficult.

3.1 Esterolytic antibody 17E8

The mechanism of action of several catalytic antibodies discussed in detail in the last review[1] have been revised subsequently, in light of new results. Antibody 17E8 was thought to act by a serine-protease-like mechanism involving the formation of an acyl-antibody intermediate. Evidence for this was the presence of a 'catalytic dyad' of His^{H35} and Ser^{H95} close to the phosphonate of the hapten as identified by the X-ray crystal structure of the hapten–Fab complex, and kinetic data. However, site-directed mutagenesis of Ser^{H95} to an alanine residue did not abolish the catalytic activity as expected, but actually produced an antibody with an increased reaction specificity.[115] Replacement of His^{H35} by a glutamine halves the catalytic rate for the reaction and increases K_M by an order of magnitude, whilst substitution of the same residue by an alanine destroyed all catalytic activity. These experiments suggest that the hydrogen bonding ability of residue H35 is more important than its potential role as a general acid/general base, electrostatic or nucleophilic properties, as this is retained in the His^{H35}Gln mutant. Whether residue H35 is important for activating a water molecule, as has been proposed in the closely related antibody 29G11 obtained from the same immunisation,[93,116] or whether it is involved in oxyanion stabilisation remains unclear. A mutagenesis study involving so-called 'second sphere' residues (residues that are not in direct contact with the hapten, but may influence the folding of the hypervariable loops) has been conducted by Arkin and Wells[117] and they have identified a double mutant (Tyr^{H100A} to Asn; Tyr^{H97} to Arg) which increases

the catalytic turnover three- to four-fold whilst also lowering the Michaelis constant by three- to five-fold.

3.2 Esterolytic antibody 48G7

Esterolytic antibody 48G7 was the first to have its germline antibody reproduced. In this case it was found that none of the nine somatic mutations that had occurred were in direct contact with the antigen, but rather they 'firmed up' the hypervariable loop conformations reducing the polyselectivity of the binding site. The impact of these mutations could be studied further with the elucidation of the crystal structure of the germline antibody Fab complexed with the hapten.[99,100] It is evident that the differences in structure between the free and bound germline antibody are significantly greater than those of the mature antibody in this case. Whilst the mature antibody is preorganised to form a suitable binding pocket for the transition state analogue, the germline antibody has to substantially reorganise to establish a similar number of interactions. This accounts for a significant part of the 30,000 factor difference in affinity between the germline and mature antibodies, the only direct change being the addition of a hydrogen bond from Tyr^{H33} to the phosphonate oxyanion.

3.3 Amido- and esterolytic antibody 43C9

Antibody NPN43C9 has recently been crystallised as a scFv (single-chain variable fragment)[103] allowing a direct comparison with a previously generated computer model.[102] Unfortunately co-crystals were only obtained with *p*-nitrophenol rather than the complete hapten. However, the computer model appears to have given a fair representation of the antibody structure apart from the unusual conformation of the difficult to predict H3 loop. In the crystal this forms an extended β-sheet producing a deeper binding pocket than expected. As predicted by the model, Arg^{L91} is well positioned to participate in oxyanion stabilisation, and His^{L91} as a nucleophile. The crystal structure also suggests that two tyrosine residues in close proximity to His^{L91} may improve its nucleophilicity.

3.4 Esterolytic antibodies D2.3, D2.4 and D2.5

Knossow and co-workers have solved the structures of three esterolytic antibodies from the same immunisation.[96] Antibodies D2.3, D2.4 and D2.5 were three of nine catalytic antibodies identified from 1570 hybridomas using catELISA (Catalytic Enzyme-linked Immunosorbent assay). Whilst the rate acceleration and specificity constants for the three antibodies vary by three orders of magnitude, the number of hydrogen bonds (5 in each case) and van der Waals contacts (86, 96 and 76 respectively) with the hapten are similar. The sequences of D2.4 and D2.5 differ from those of D2.3 at 17 and 15 positions respectively, with the main differences being in loop H3. The H3 loop for antibodies D2.3 and D2.4 have arisen from different joining recombinations of the D and J genes which result in an insertion of an extra residue in the H3 loop of D2.4. The framework regions of these three antibodies superpose. Catalysis is thought to be due primarily to stabilisation of the oxyanion generated during the reaction. In the case of antibody D2.3 this is thought to be due to interactions with Tyr^{H100d} and Asn^{L34} which are within hydrogen bonding distance in the Fab-hapten complex. The second phosphonyl oxygen is positioned to interact with the hydroxy of Trp^{H95} and a water molecule. Residues Tyr^{H100d} and Asn^{L34} are retained in antibody D2.4, whilst Asn^{L34} is replaced by a serine in D2.5 which may account for its poorer catalytic activity, as this residue is too far away from the phosphonyl oxygen to bind directly, but is capable of interacting *via* a water molecule.

3.5 Oxygenation antibody 28B4

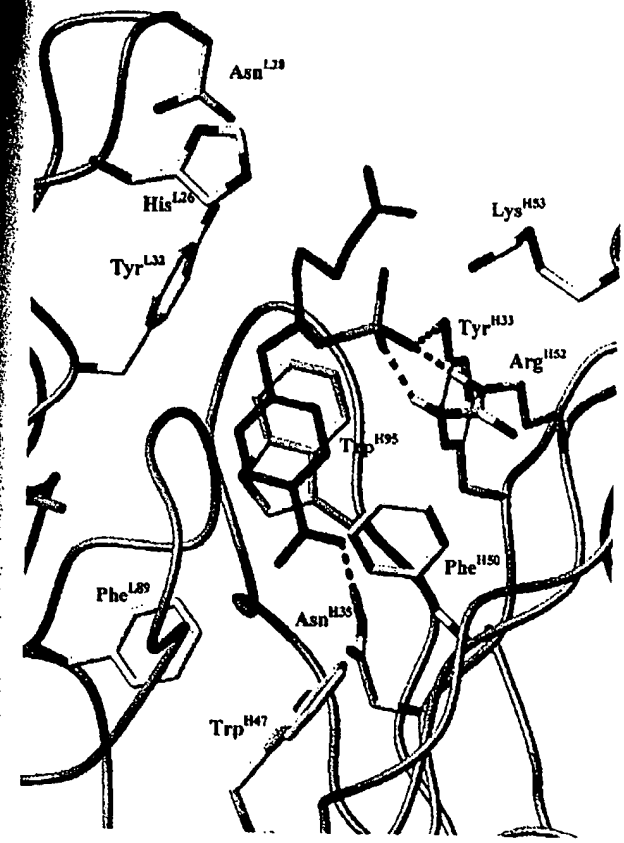
The crystal structure of the oxygenation antibody 28B4 generated using a hapten containing a phosphonate group has also been solved (Fig. 1upper).[106] The antigen binding cleft is ~10 Å deep and 7 Å wide in this case with the *p*-nitrophenyl group of the hapten being buried at the bottom of the pocket and the phosphonate near the surface. There are 11 van der Waals interactions and four hydrogen bonds between the hapten and the antibody. Hydrogen bonds, salt bridges and electrostatic interactions are primarily between the phosphonate and residues Tyr^{H33}, Arg^{H52}, and Lys^{H53}. This antibody shares the same germline family of V_H genes as the phosphocholine binding antibody McPC603, with the phosphonate binding site performing as a periodate binding pocket in the catalytic reaction (K_M = 252 μmol dm^{-3}). The *p*-nitrophenyl ring is deeply embedded in a hydrophobic cavity consisting of residues (Trp^{H95}, Tyr^{H33}, Trp^{H47}, Phe^{L89} and Phe^{H50}), being sandwiched between residues Trp^{H95} and Phe^{H50} whilst the nitro group hydrogen bonds to Asn^{H35}. The ammonium ion of the hapten appears to be stabilised by interactions with the π-system of Tyr^{L32}, a similar amino–aromatic interaction to that found in McPC603.[104]

It is proposed that the phosphonate binding residues stabilise the developing negative charge on the periodate ion, whilst the π-system of Tyr^{L32} stabilises the developing positive charge on the sulfur of the substrate in the oxygenation transition state, whilst the binding pocket topology correctly orientates the two reactants.

3.6 Pericyclic catalysts (AZ-28, 39-A11, 13G5)

A number of antibodies that catalyse pericyclic rearrangements have been reported in addition to the chorismate mutase antibody described in the previous review.[108] Ulrich and Schultz have examined the activity of antibody AZ-28 which catalyses the oxy-Cope rearrangement (Fig. 1 lower).[111] Interestingly when they produced the germline version of this antibody which required the replacement of Ser^{L34} with Asn and Ala^{L51} with Thr, they found that the antibody affords a 35-fold rate enhancement compared with the mature form.[112] This is rationalised in terms of the mature antibody having a more rigid binding pocket which rotates the cyclohexadiene core of the substrate out of the chair conformation preferred by the oxy-Cope reaction and hence reduces orbital overlap between adjacent centres. It is thought that the antibody acts by a combination of entropic and electronic effects. The binding site preferentially binds the chair conformation of the substrate and hence acts as an entropic sink, increasing the frequency of substrate molecules in the correct geometry. It is also thought that His^{H96} and Glu^{H50} may hydrogen bond with the hydroxy group of the substrate increasing the oxygen electron density and so electronically assisting the reaction. Overlap between the π-orbitals of the aryl substituents and diene are also thought to increase the rate of the rearrangement. This combination of stereoelectronic effects results in the rate acceleration observed. This reaction does not follow the rule of rate enhancement being equal to differential binding ($k_{cat}/k_{uncat} = K_I/K_M$), as this does not take into account the additional assistance of the electronic factors that are brought into play here.

Three antibodies capable of catalysing Diels–Alder cycloadditions have also had their crystal structures solved.[109–111] In the case of antibody 39-A11 structures of both the Fab·hapten complex of the mature and germline precursor antibodies have been determined. The binding pocket is found to be ~9 Å wide and 12 Å deep, with 89 van der Waals interactions and 2 hydrogen bonds being identified with the hapten (Fig. 2 upper). As for the Cope rearrangement discussed above, the primary role of the antibody in catalysis appears to be binding the diene and dienophile in a reactive conformation as programmed by the bicyclo[2.2.2]octene hapten. Interestingly, mature antibody 39-A11 is only two somatic mutations away from its germline precursor, a con-



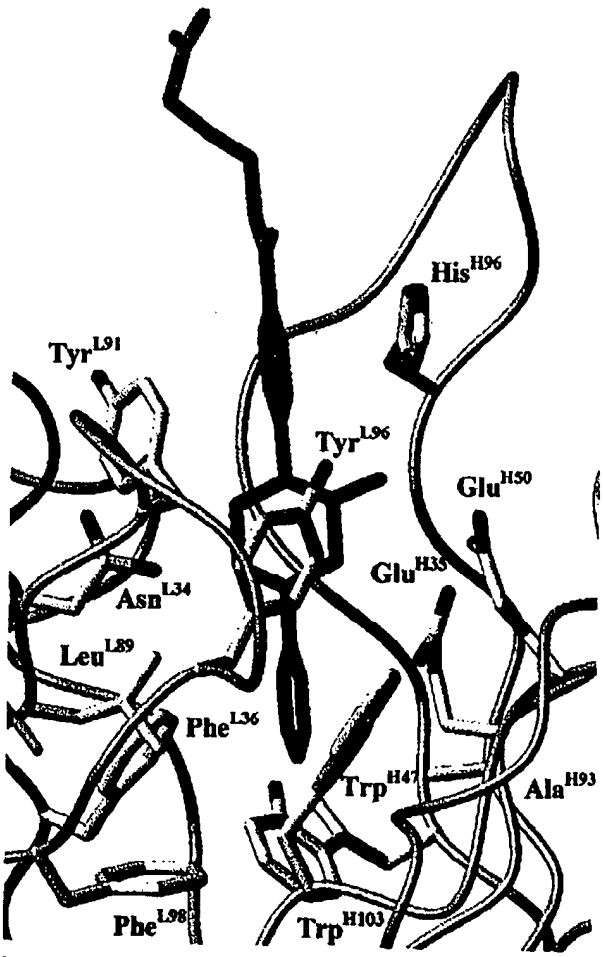
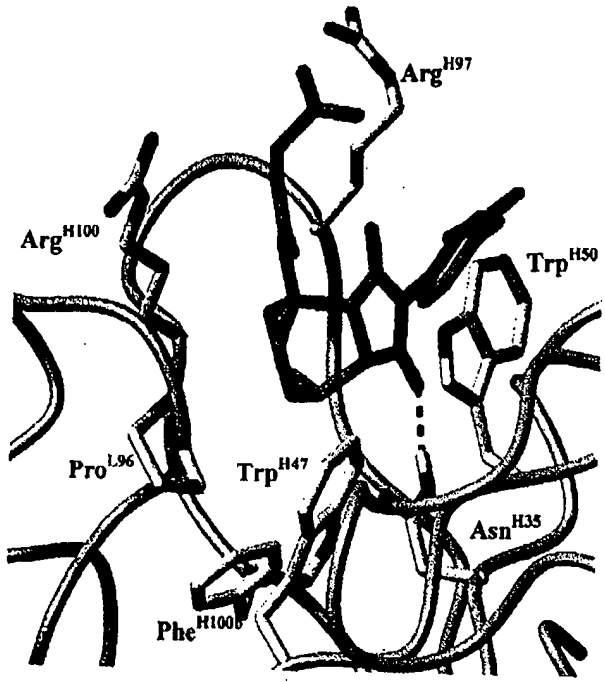


Fig. 1 Antibody binding sites with haptens bound: (upper) Oxygenation antibody 28B4; (lower) oxy-Cope antibody AZ-28.



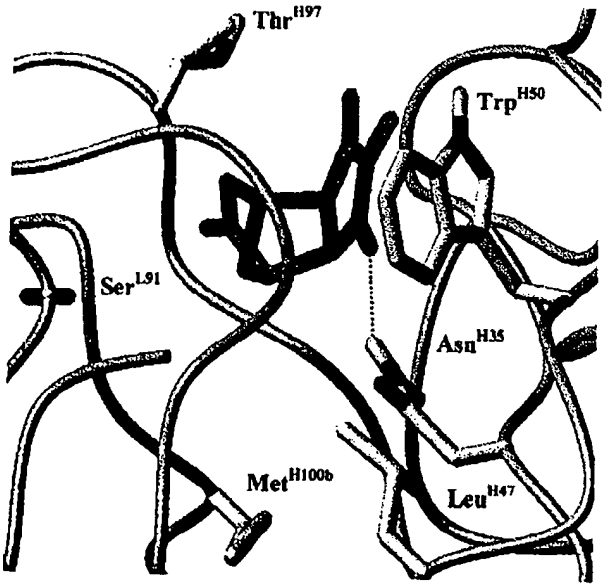


Fig. 2 Antibody binding sites with haptens bound: (upper) Diels–Alder antibody 39-A11; (lower) Diels–Alderase antibody 7G12 1E9.

servative mutation in CDRL1 Val^{L27c} to Leu, and a more important mutation Ser^{L91} to Val in CDRL3 which accounts for the higher affinity of the mature antibody for its hapten than the germline precursor (K_d = 10 and 379 nmol dm^{-3} respectively). These differences in binding to the hapten are reflected in the k_{cat} (0.67 s^{-1} *cf.* 0.17 s^{-1}) and K_M (1200 μmol dm^{-3}(diene), 740 μmol dm^{-3}(dienophile) for 39-A11 compared with 1200 μmol dm^{-3} and 450 μmol dm^{-3} for germline antibody). Antibody 39-A11 is also very similar in sequence to anti-testosterone antibody DB3, anti-peptide antibody (TE33) and anti-hexachloronorbornene (Diels–Alderase) antibody (1E9). This highlights the fact that only a few key mutations are required to go from a polyspecific germline antibody which can be selected for clonal expansion and affinity maturation by a wide range of antigens to one with high specificity for a single hapten. The catalytic activity of 39-A11 has been enhanced by improving the interactions between the diene and antigen binding site.[118] Substitution of Val^{L91} (Ser in germline) with aromatic amino acids that are capable of π–π

stacking with the diene (Phe/Tyr) (Val^{L91} to Tyr: $k_{cat} = 6.3\ s^{-1}$, $K_d = 4$ nmol dm^{-3}, K_M(diene) 2100 μmol dm^{-3}, K_M(dienophile) 420 μmol dm^{-3}) increases the K_M for the diene, but decreases the K_d for the hapten, demonstrating that the mutant preferentially binds the reaction transition-state rather than the substrate ground states. This is reflected in rate acceleration of these mutants, which are an order of magnitude faster than 39-A11. Whilst a mutation of Pro^{L96} to Tyr also had a similar effect, the two mutations were found not to be additive.

As indicated above the most efficient Diels–Alderase antibody, 1E9 generated against a hexachloronorbornene hapten is closely related in sequence to 39-A11.[113] This antibody differs from its putative germline genes *VFM11* and V_K5.1 by eight somatic mutations in V_H and six in V_L. The hapten is 86.3% buried in a hydrophobic pocket, which contains only two polar residues Asn^{H35} and Ser^{L91}. The authors describe the shape complementarity of the binding pocket as being a "hand-in-glove like fit" due to the low gap volume between the hapten and binding pocket surface (Fig. 2 lower). This is much less than is found in the other pericyclic antibody catalysts, partly due to the somatic mutation of Ser^{L89} to Phe which fills a prominent cavity found in antibodies 39-A11 and D83. The hapten makes 121 van der Waals contacts with the antibody and a hydrogen bond from Asn^{H35} to one of the succinimyl carbonyls. One edge of the hapten is solvent exposed, especially around the bridgehead chlorine. This has allowed substrate mutagenesis to be used to test a more water soluble thiophene dioxide methoxy acetate. A better estimate of the effective molarity (k_{cat}/k_{uncat}) for the Diels–Alder reaction was then determined. This was found to be 1000 mol dm^{-3} which is 10^2–10^3-fold higher than that for the other Diels–Alderases. Similar to other pericyclic antibody catalysts, 1E9 does not appear to function as an entropy trap as initially thought, but rather the rate acceleration derives from a lowering of the enthalpic barrier. Desolvation has also been invoked to account for the rate acceleration observed, but again this Diels–Alder reaction is retarded in acetonitrile relative to water. This leaves the possibility of favourable electrostatic interactions in the transition-state relative to the ground state, and a shortening of the hydrogen bond between the dienophile and His^{H35}.

Janda and co-workers reported one of the more unusual hapten designs in the use of a ferrocene derivative to generate antibody 13G5, which preferentially catalyses an *exo*-Diels–Alder reaction to give the *ortho*-adduct as the major product.[109] The transition-state for the formation of this compound has been predicted to be 2–4 kcal mol^{-1} lower than that of the *meta*-adducts, but 1.9 kcal mol^{-1} higher than the preferred *ortho-endo*-adduct. This antibody demonstrates the rerouting of a reaction *via* a normally disfavoured transition state. Perhaps more interestingly it also demonstrates that a conformationally flexible hapten can be used successfully as a hapten for a geometrically demanding reaction.

The ferrocenyl hapten makes three hydrogen bonds and 45 van der Waals contacts with the antibody binding site (Fig. 3 upper). The hydrogen bonds are between the oxygen of the (dimethylamino)-carbonyl of the hapten and the hydroxy of Tyr^{L36}, and the ferrocenyl carboxylate oxygens and the side chains of Asp^{H50} and Asn^{L91}. These hold the 1 and 1′ ferrocene substituents in an almost eclipsed conformation as opposed to the *anti*-conformation seen for the hapten in solution. The cyclopentadiene rings are also prevented from rotation by the sidechains of Gly^{H35} and Ala^{H93}.

Examination of the antibody binding site and docking of the putative transition state structures indicated that the (3*R*,4*R*)-*exo* transition state isomer is preferentially bound. Residue Tyr^{L36} acts as a Lewis acid activating the dienophile, and Asn^{L91} and Asp^{H50} form hydrogen bonds to the carboxylate of the diene, both activating and orientating it for the *exo*-Diels–Alder reaction.

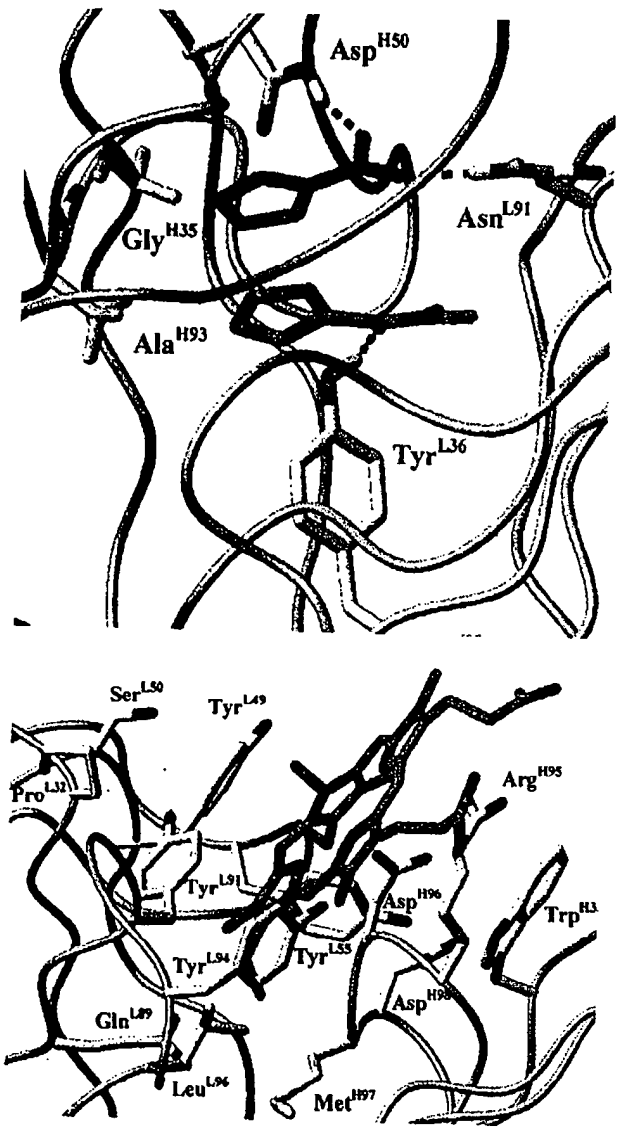


Fig. 3 Antibody binding sites with haptens bound: (upper) Diels–Alder antibody 13G5; (lower) Chelatase antibody 7G12.

3.7 Chelatase antibody 7G12

N-Methylmesoporphyrin was first used as a hapten to generate an antibody with peroxidase activity.[119] The same antibody was then shown to catalyse the insertion of copper(II) into a mesoporphyrin,[9] a reaction reminiscent of that catalysed by ferrochelatase (EC 4.99.1.1), an enzyme which catalyses the insertion of ferrous ions into protoporphyrin IX, which is an essential step in heme biosynthesis. The structures of both the cuprochelatase antibody (Fig. 3 lower) and ferrochelatase enzyme have been solved by X-ray crystallography (the ferrochelatase with porphyrin bound only very recently (PDB: 1D0Z, 1C9E, 1C1H)).[120] The levels of distortion of the porphyrin ring in both the enzyme and antibody have been studied by Raman spectroscopy.[121]

The *N*-methylmesoporphyrin hapten which has three coplanar pyrroles and one distorted out of plane by the *N*-methyl substituent is buried edge-on into an asymmetric shallow cleft generated by the H1 and H3 loops on one face of the porphyrin and the L3 CDR which only partially covers the other face, leaving two of the pyrroles exposed to solvent. The hapten-binding pocket is lined with hydrophobic residues Tyr^{L30}, Leu^{L46}, Tyr^{L49}, Tyr^{L55}, Gln^{L89}, Tyr^{L91}, Tyr^{L94}, Leu^{L96}, Trp^{H33}, Met^{H50} and Met^{H97}, whilst the terminal carboxylate oxygen of Arg^{H95} is positioned 1.9 Å from the centre of the

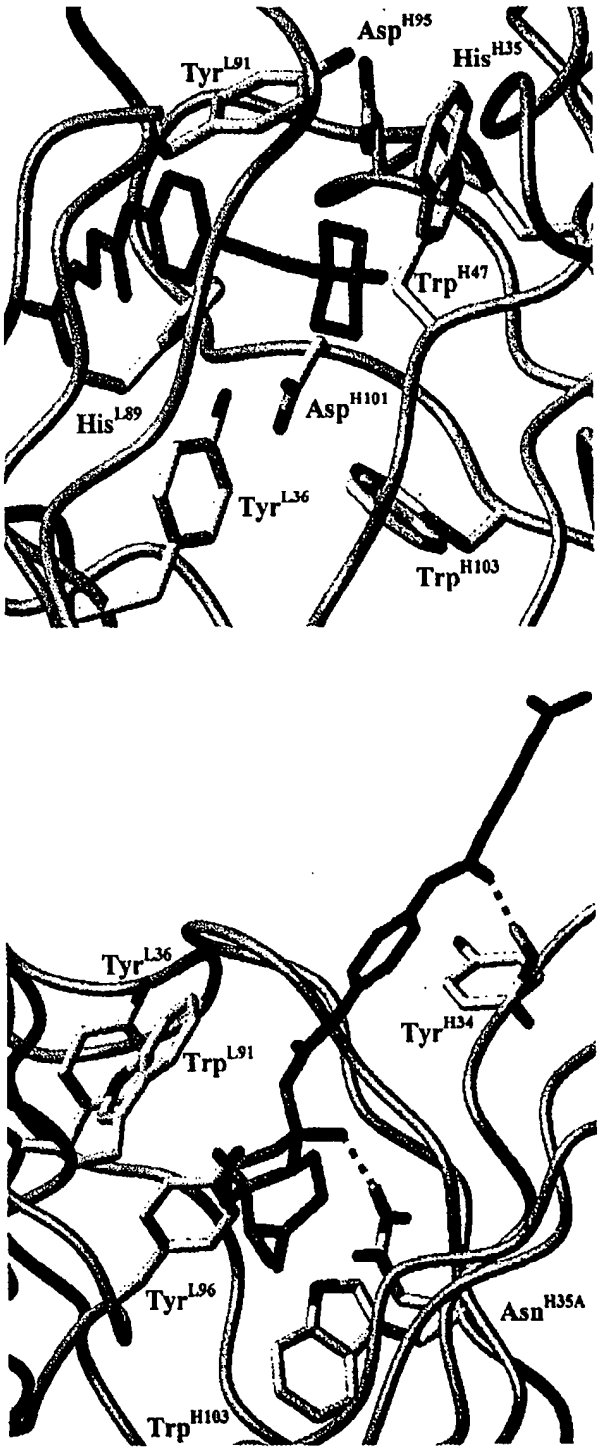


Fig. 4 Antibody binding sites with haptens bound: (upper) Cyclisation antibody 5C8; (lower) Cyclisation antibody HA5 19A4.

porphyrin through a hydrogen bond between it and the adjacent Asp^{H95}. In all, 114 van der Waals contacts are made between the hapten and antibody. Mutagenesis studies have shown that Asp^{H96} is essential for catalysis, and it has been proposed that it acts either as a base to remove protons from the mesoporphyrin substrate prior to copper(II) insertion, or as a transient ligand for the copper(II) (a histidine fulfils these roles in the ferrochelatase). The germline antibody sequence has been identified for antibody 7G12 and indicates that affinity maturation has caused two mutations in the light chain (Ser^{L14} to Thr; Ala^{L32} to Pro) and three in the heavy chain (Asn^{H50} to Met; Ser^{H76} to Asn; Ser^{H97} to Met). Studies on the 7G12 antibody, in which individual mutations were made, reveal that a Pro^{L32} to Ala mutant maintained its k_{cat} value (37 h^{-1}), but exhibited a significantly increased K_M (400 μmol dm^{-3}). The Met^{H97}Ser mutant had a significantly reduced k_{cat} (2.2 h^{-1}), but similar K_M (190 μmol dm^{-3}) suggesting that the mutation is important for increased recognition of the transition-state (hapten structure) over the ground state (substrate structure).

3.8 Cyclisation catalysts (5C8, 19A4)

The rerouting of a cyclisation reaction to give a disfavoured product was discussed in detail in the previous review,[1] at which time antibodies 26D9 and 17F6 had been shown to catalyse the 6-*endo*-*tet* cyclisation of an epoxy alcohol, which is normally disfavoured in solution. The hapten for these antibodies included a neutral *N*-oxide piperidine. In a more recent study, antibodies have been raised against an *N*-methylpiperidinium hapten in order to determine if a positively charged hapten would elicit a better catalyst. Antibodies 5C8 and 14B9 were isolated from these immunisations and found to catalyse the *endo*-*tet* cyclisation.[122] The structure of the Fab of 5C8 in complex with both *N*-oxide and *N*-methylpiperidinium haptens has been solved (Fig. 4 upper), and molecular modelling studies involving the docking of the substrate molecules in the antibody binding pocket have been undertaken using Autodock.[123] The binding site for these haptens unusually involves a large number of framework region residues (Phe^{L98}, Val^{H37}, Trp^{H47}, Ala^{H93}, Trp^{H103}), the phenyl ring of the hapten is sandwiched between His^{L34} and Pro^{H96}, the pyridinium ring by a pocket involving Trp^{H47}, Tyr^{L91}, and His^{H35}. The quarternary nitrogen is sandwiched between two aspartic acid sidechain carboxylates Asp^{H95} and Asp^{H101} which are 3.7 Å and 4.0 Å away respectively. These make either hydrogen-bond or electrostatic interactions with the hapten. The binding site is rich in proton donors/acceptors (His^{L34}, His^{L89}, His^{H35}, Asp^{H95}, Asp^{H101}, Tyr^{L36}, Tyr^{L91}). The mechanism of action of this antibody has been proposed based on a combination of the conservation of residues in catalytic antibodies when compared with non-catalytic antibodies, the stereoselectivity of the antibody, and the postulated pK_a's of the active site residues. The favoured mechanism involves His^{L89} acting as a base to deprotonate the hydroxide which then attacks the epoxide which is activated by hydrogen bonding to the carboxylic acid of Asp^{H95} (Scheme 36). An alternative mechanism involving Tyr^{L36} acting as the base appears less likely especially from studies in which the epoxy alcohols are docked in the binding site.

More recently, the X-ray crystal structure of antibody HA5 19A4 has been solved (Fig. 4 lower).[63] This antibody catalyses the cyclisation of a polyene to give a mixture of *trans*-decalins. The hapten again contains a nitrogen-oxide moiety to function in a 'bait & switch' fashion to elicit groups, which would facilitate the protonation of the leaving group. This is manifested by the occurrence of a hydrogen bond between the sidechain of Asn^{H35A} and the oxygen of the *N*-oxide, and the proximity of the faces of the sidechains of Trp^{L91} and Tyr^{L96} whose π-electron clouds stabilise the adjacent positively charged nitrogen through electrostatic interactions.

3.9 Aldolase antibody 33F12

Antibody 33F12 catalyses aldol condensation reactions on a wide range of substrates *via* a Schiff base intermediate involving an active-site lysine. The antibody has been crystallised in the absence of a hapten/inhibitor, and the catalytic Lys identified as Lys^{H93} based on the conservation of this residue in another aldolase antibody, 38C2.[48] This lysine is buried in an 11 Å deep pocket and is surrounded by a number of hydrophobic residues (Leu^{H4}, Met^{H34}, Val^{H37}, Cys^{H92}, Ile^{H94}, Tyr^{H95}, Tyr^{H102}, and Trp^{H103}) all of which are conserved, with the exception of

Ile^{H94} which is a Thr in 38C2. This hydrophobic microenvironment is thought to lower the pK_a of Lys^{H93} from its solution value of 10.5 to ~6.0 discouraging its protonation and allowing it to act as an efficient nucleophile at neutral pH. In this way it is reminiscent of the pH dependence of catalysis of FDP aldolase which is proposed to have a similar mechanism of action. However, in the latter case the occurrence of multiple lysines is thought to be the reason for the presence of one lysine with an usually low pK_a. Antibodies 33F12 and 38C2 are highly homologous only differing by 10 residues in V_L and 9 in V_H. The CDRH3 sequence being identical in both. This suggests that they share a germline ancestor (although as our current knowledge of the murine germline repertoire is incomplete, this is not definite).

Lerner has also examined the structure of antibodies 40F12 and 42F1 that were generated against a sulfone diketone and have similar enantioselectivities to 33F12 and 38C2 generated against a diketone hapten.[124] Antibodies 40F12 and 42F1 are highly homologous only differing by 3 residues in V_L and 2 in V_H suggesting that they are somatic variants. The 40F12/42F1 family uses a CDRH3 that is 5 amino acids longer than the 33F12/38C2 family, but other than that the V_H sequences of both appear to have arisen from the V_H22.1 gene segment. The V_L sequences are significantly more divergent. Most importantly, Lys^{H93} appears to have been introduced as a somatic mutation in all four antibodies. Mutagenesis of this residue in 40F12 and 33F12 abolishes any aldol or retro-aldol activity, indicating this residues essential role in catalysis.

The binding pockets of 40F12/42F1 have similar hydrophobic character to those of 33F12/38C2. Again this non-polar environment is thought to be responsible for perturbing the pK_a of the ε-amino group of the lysine to 6.0.

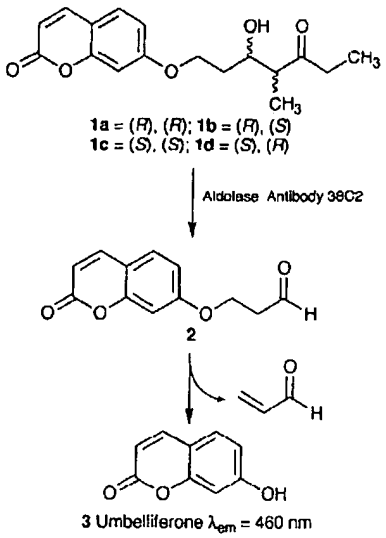
4 Advances in screening and selection of catalytic antibodies catalysts

Potential antibody catalysts are identified initially on their ability to bind a transition state analogue, based on the proposed link between transition state stabilisation and catalytic proficiency.[125] Those antibodies that are catalytically active are then identified by monitoring product formation upon incubation with the relevant substrate. While assaying for binding affinity using an immunoassay is both rapid, and can be performed as an automated, high throughput operation, the subsequent identification of catalytic activity is more laborious. The method used is very dependent on both the quantity of antibody available and the type of reaction being sought. One of the most active areas of research in catalytic antibody technology recently has been the development of rapid and sensitive assays for the early identification of antibody catalysts. The methods reviewed here are also applicable to the detection of nucleic acid catalysts, or new enzyme activities generated by directed evolution.

4.1 Fluorogenic substrates

The ability to monitor product formation spectroscopically offers a rapid and sensitive assay for the early identification of active catalysts. Reymond *et al.* have employed fluorogenic substrates which liberate a fluorescent product to detect antibodies which catalyse either a retro-aldol reaction,[126] or a retro-Diels-Alder reaction.[127]

Aldolase substrates **1a–d** (Scheme 38) were designed to liberate the fluorescent product umbelliferone **3** following conversion to the unstable aldehyde intermediate **2** by aldolase antibodies. Aldehyde **2** rapidly undergoes β-elimination, preventing reformation of the aldol product. The accumulation of umbelliferone is then readily measured by monitoring fluorescence increase at 460 nm (excitation at 360 nm). In addition, the stereoselectivity of the antibody catalyst can be



Scheme 38

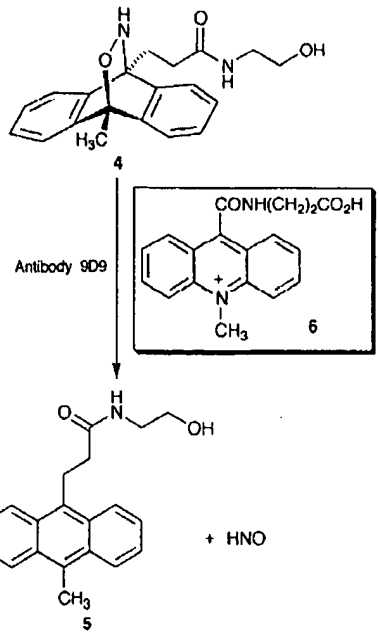
probed by using the four possible stereoisomeric substrates in separate assays. The use of this assay was demonstrated with the aldolase antibody 38C2 (see section 2.11).[44] An (*S*)-enantioselectivity at the β-OH position was observed as only aldols **1c** and **1d** were substrates for the antibody. An *anti*-aldol diastereoselectivity was also observed with **1c** being the favoured substrate.

Similarly, the accumulation of the fluorescent anthracene derivative **5** was used to identify antibodies which catalyse the retro-Diels–Alder reaction of **4**, releasing **5** and nitroxy (HNO) (Scheme 39).[127] More than 14000 samples from a total of 11 immunisations were assayed using this substrate system, in conjunction with ELISA binding assays. A number of related transition state analogues were also used to assess the impact of hapten structure. In addition a number of heterologous immunisations using multiple haptens were also carried out. From 14000 candidates, 8 active antibodies were identified, capable of rate accelerations (k_{cat}/k_{uncat}) varying from 10 to 2500. As the catalytic assays were carried out directly on unpurified hybridoma cell culture supernatant, identification of potential catalysts was possible at a very early stage. The number of hybridoma cell lines, which had to be maintained, could therefore be reduced rapidly, and as a result much larger numbers of antibody samples could be screened for activity under a variety of conditions.

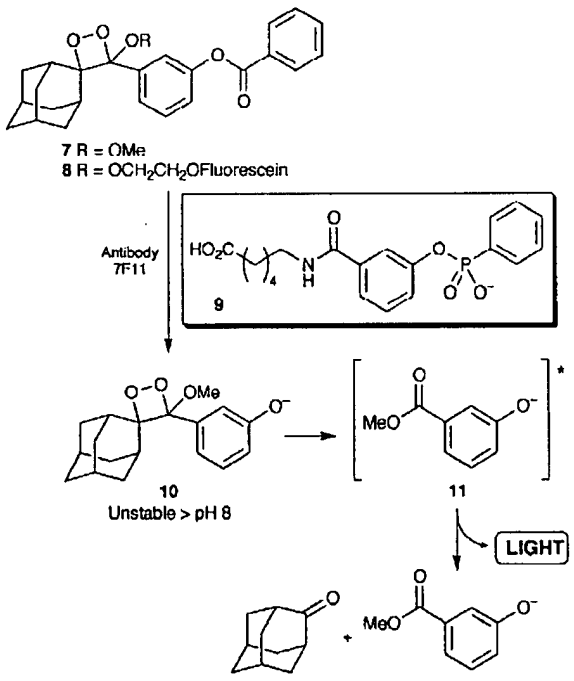
4.2 Chemiluminescent substrates

Chemiluminescent enzyme assays have found increasing favour in clinical laboratories due to their high sensitivity, low background interference and operational simplicity.

A range of substrate systems have been developed in recent years in which this process of light emission can be selectively triggered as a result of enzyme modification.[128,129] A number of these enzyme substrates are now available commercially.[130] Thomas and co-workers chose to adapt the hydroxyaryl substituted adamantyl-1,2-dioxetanes, developed by Schaap *et al.*[131] to screen for hydrolytic catalytic activity.[132] These are a highly versatile class of chemiluminescent substrates, which undergo Chemically Initiated Electron Exchange Luminescence (CIEEL) following cleavage of a triggering group (Scheme 40). This triggering group, at the key 3-hydroxyaryl position, can be varied according to the desired enzyme activity (ester, carbamate, or phosphate or glycoside hydrolysis). As a result of enzyme cleavage the phenolate product **10** undergoes fragmentation across the 1,2-dioxetane ring to generate a singlet excited ester fragment **11**. This fragment relaxes to its ground state



Scheme 39



Scheme 40

with the emission of light, with a quantum yield (ϕ) ~ 10^{-6}. Antibodies generated against the phosphonate transition state analogue **9** were screened for their ability to hydrolyse benzoate ester **7** in a microtitre plate assay. An active catalyst, 7F11 was rapidly identified which triggered a glow-type light emission from a 100 μmol dm^{-3} solution of dioxetane **7** in aqueous buffer at pH 9.0. Kinetic analysis of this antibody was carried out, and the catalyst was shown to obey saturation kinetics with $k_{cat} = 0.02\ s^{-1}$, $K_M = 2.5$ μmol dm^{-3} and $k_{cat}/k_{uncat} = 2 \times 10^6$. The sensitivity of the chemiluminescence assay was increased by adding fluorescein to the assay solution. Intermolecular energy transfer from the excited ester fragment to fluorescein resulted in enhanced light emission. The quantum yield was increased further by making this energy transfer an intramolecular process by tethering the fluorescer to the substrate molecule itself.[133] The resulting increases in chemiluminescence allowed significantly lower concentrations of catalyst to be detected, making this assay a viable strategy for screening hybridoma culture supernatants directly for catalytic activity.

4.3 Competitive immunoassays

The adaptation of the conventional ELISA to detect catalytic activity, rather than simple binding activity, was first demonstrated by Tawfik *et al.* who termed this technique catELISA (catalytic ELISA).[134] This process uses an anti-product antibody to detect the formation of reaction products following incubation with the catalytic antibody. For detection purposes, the substrate was tethered to a carrier protein and immobilised on the surface of microtitre plate, formation of the product–antibody complex was then visualised using a secondary antibody–enzyme conjugate, as in conventional ELISA. This process exploited the high sensitivity of antibody based detection systems, for which immunoassays are well known.

One of the problems with catELISA, however, is the requirement that the substrate has to be present in an immobilised form. The catalysis itself is therefore carried out on the solid phase. This has several potential setbacks, firstly that the substrate molecule itself has to include a linker group for covalent attachment to a carrier protein, thus complicating its synthesis. Secondly, recognition of the modified substrate by the catalytic antibody may then be affected, particularly in the case of small, weakly immunogenic substrates. The determination of kinetic constants is also hampered by the inability to accurately determine the immobilised substrate concentration.

In an attempt to overcome the problems associated with catELISA, two independent research groups have adapted the competitive enzyme immunoassay (EIA) format for the detection of catalytic activity.[135,136] In this approach, the desired substrate is incubated with the catalytic antibody free in solution. The product is then captured on the solid surface using an anti-product antibody, which is in turn captured by a secondary antibody. Quantification of the product concentration is achieved using an enzyme labelled product, which competes with the free product for the immobilised anti-product antibody binding sites. The measured immobilised enzyme activity is then inversely related to the concentration of product present (Fig. 5). By generating a calibration curve with known concentrations of product, the concentration of product present in a test reaction can be accurately determined. In addition, kinetic analysis can be carried out by removing samples at different times and capturing the product for quantification.

Benedetti and co-workers initially set out to detect the hydrolysis of esters such as **12** using a catELISA assay (Scheme 41).[135] This proved unsuccessful when the uncatalysed hydrolysis of **12a** at pH 11.7 could not be observed, even though this reaction was shown to proceed with a rate constant in solution of $1.16 \times 10^{-4}\ s^{-1}$, as monitored by HPLC. The authors suggested that this was due to the hydrophobic environment in which the substrate was immobilised. To overcome this problem, the competitive catELISA assay was developed. Using rabbit serum against the acid product **13**, and a conjugate of **13** with the enzyme horseradish peroxidase, the uncatalysed hydrolysis of **12a** was successfully monitored using the competitive catELISA technique. The kinetic data that was generated fitted well with rate data obtained using a HPLC assay. The authors then demonstrated the application of this assay in monitoring biocatalyst activity by screening a range of common esterase enzymes for activity with substrates **12a** and **12b**. Pig and horse liver esterases were both shown to hydrolyse **12b**. No catalytic antibodies were reported in this study, but the authors defined the minimum rate of hydrolysis which could be detected as $k_{cat} = 5 \times 10^{-7}\ s^{-1}$. This is significantly less than

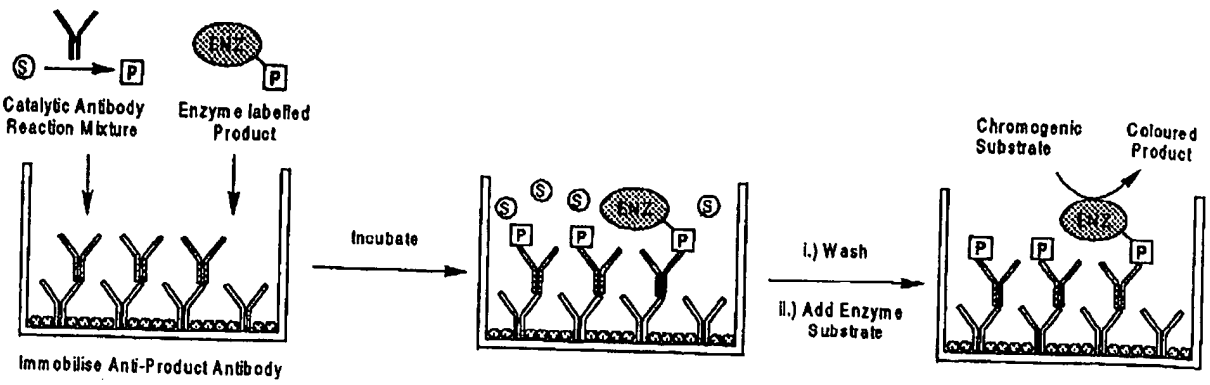
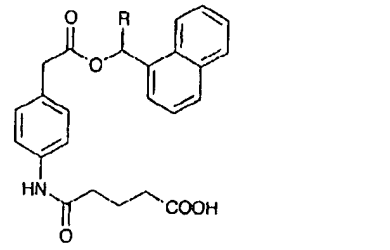
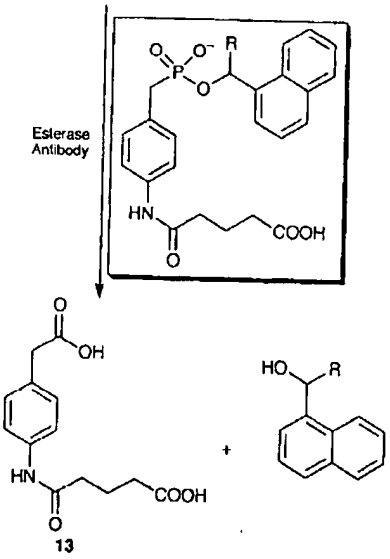
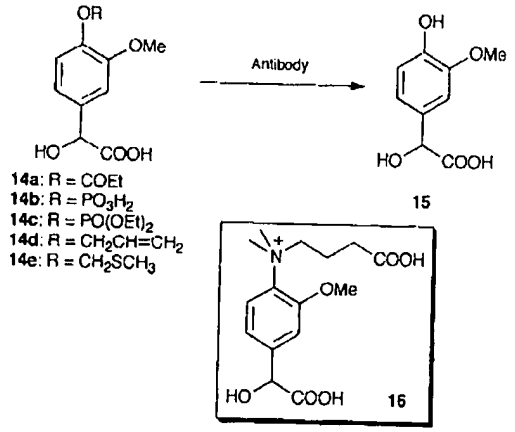


Fig. 5 Detection of reaction products using a competitive catalytic ELISA assay.





Scheme 41



Scheme 42

the k_{cat} values typical of catalytic antibodies, indicating that this assay could be used in the identification of hydrolytic antibodies.

In parallel to this study, Taran *et al.*[136] applied a competitive catELISA assay to the screening of a set of monoclonal antibodies for hydrolytic activity, using a range of potential substrates (Scheme 42). As the hydrolytic cleavage of all five substrates results in the formation of the common phenolic product **15**, anti-product antibodies were produced against hapten **15**. A conjugate of **15** with the enzyme acetylcholine esterase was also produced as a labelled competitor ligand. The cationic hapten **16** was used to obtain potential catalytic antibodies by charge complementation to induce catalytic residues in the antibody. Using the competitive catELISA assay, three esterolytic abzymes were identified which hydrolysed substrate **14a**. Rate accelerations (k_{cat}/k_{uncat}) of 570 (H3–12), 160 (H3–15) and 480 (H3–32) were observed. These activities were confirmed using a HPLC assay, and were shown to be specific by inhibition with hapten **16**. In addition, an antibody (H3 32) was identified which hydrolysed the thioacetal substrate **14e** in the presence of $MgCl_2$. This is the first antibody reported with this activity. No catalysts were identified which could hydrolyse substrates **14b, c** or **d**.

4.4 Selection by covalent trapping

The approaches discussed so far have employed *screening* methods to identify antibody catalysts. While these strategies have been successful when applied to antibodies obtained from hybridomas, the advent of phage-displayed antibody libraries has required the development of new methods of identifying catalysts by *selection* approaches. Briefly, the technique of phage-display (which has been discussed in greater detail in a number of reviews elsewhere[1,137]) involves the production of antibody fragments as protein-fusions with a viral coat protein. The antibody fragments are then displayed on the surface of the phage particle in a functional form, allowing their binding activity to be directly probed by affinity selection. Using this technique, libraries of up to 10^{10} members can be rapidly generated, and antibodies with a desired binding specificity can be obtained.

To screen every member of a phage library for catalytic activity is logistically impossible. The library is therefore subjected to a selection process in which positive selection is directly linked with phage replication. With conventional antibodies the selection criterion is that of simple binding to an immobilised antigen. This process is termed biopanning, and a number of rounds of selection are usually carried out to obtain an enriched pool of high affinity phage-antibodies. However, in the case of catalytic antibodies the selection process should link the catalytic activity of the antibody with immobilisation of the phage particle. In order to achieve this, therefore, the catalytic action of the antibody has to result in the formation of a

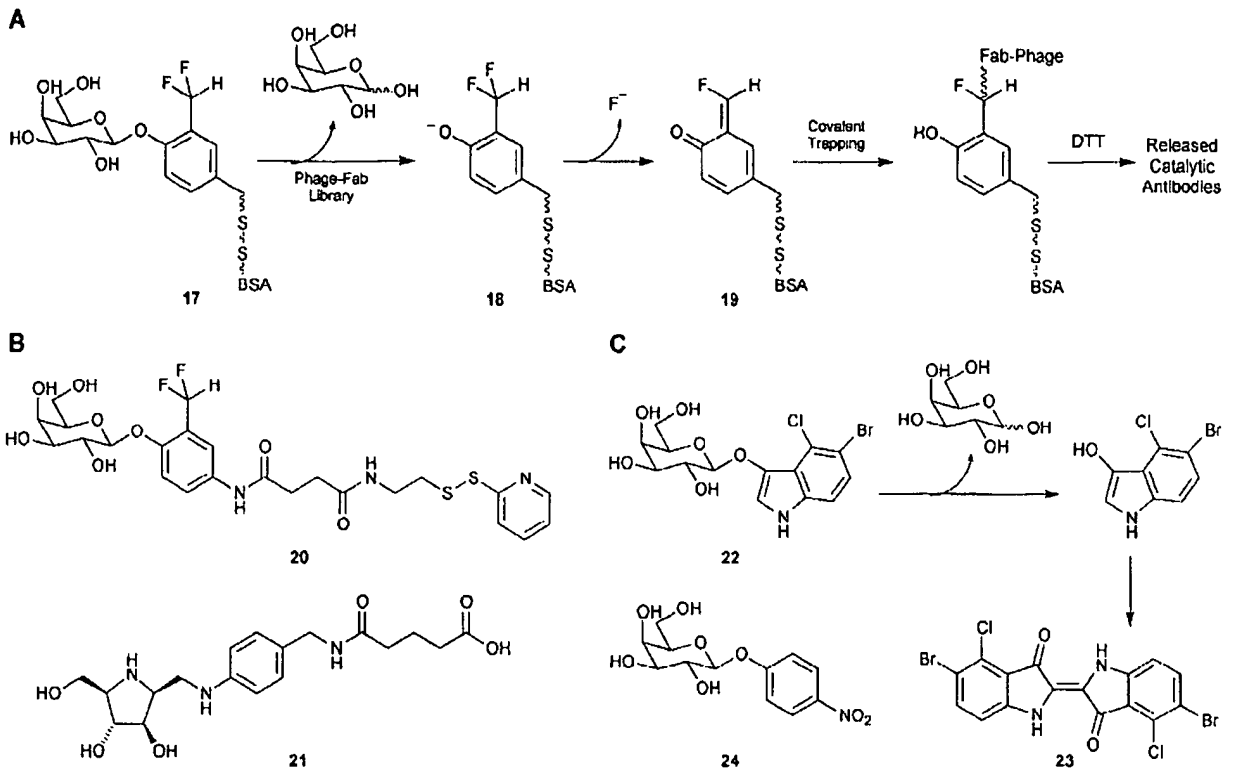


Fig. 6 Covalent trapping strategy for the selection of glycosidase phage antibodies. A mechanism of reactive quinone methide formation following antibody cleavage of difluoromethyl aryl-β-D-glucoside substrate. B Structure of mechanism based selection agent **20** and transition state analogue hapten **21** used for library generation. C Chromogenic antibody substrates used for characterisation of glycosidase activity.

covalent interaction between the phage and the immobilised selecting agent.

In an elegant approach, Janda *et al.*[138] used an immobilised mechanism-based inhibitor to selectively trap catalytically active antibodies from a library of phage displayed antibody Fab fragments. The inhibitor used was based on a difluoromethyl aryl-β-D-glucoside, which is a known β-galactosidase mechanism-based inhibitor.[139] Following hydrolytic cleavage of the galactose group in substrate **17**, the resulting difluoromethylphenol fragment **18** forms a highly reactive quinone methide species **19**. This efficiently alkylates active site nucleophiles in the protein, forming an irreversible covalent adduct (Fig. 6). The authors used this approach to search a library of antibodies for glycosidase activity. The iminocyclitol transition-state analogue **21** was used for immunisation to obtain a set of potential glycosidase antibodies from hybridomas. All the hybridomas producing hapten binding antibodies were then pooled, and a combinatorial Fab library was constructed using PCR amplification of the antibody gene sequences. This library was expressed on the surface of phage, and panned against an immobilised BSA conjugate of the mechanism-based trapping agent **20**. Four rounds of selection were carried out, with non-binders being removed by washing, and binding phage being eluted by cleavage of the disulfide linker with DTT. The catalytic activity of the selected antibody fragments was then assessed using a chromogenic assay. The antibodies were expressed in *E. coli* as soluble Fab fragments in the bacterial periplasm, and grown on culture plates containing the glycosidase substrate **22**. Active catalysts were identified by their ability to form the blue precipitate **23**.

A number of glycosidase antibodies were obtained, and a single antibody was selected for detailed kinetic analysis using the *p*-nitrophenyl-β-galactoside substrate **24**. This antibody displayed a $K_M = 530$ μmol dm^{-3} and a $k_{cat} = 0.007$ min^{-1} which represents a rate acceleration (k_{cat}/k_{uncat}) of 7×10^4. The authors then compared the antibodies they obtained using this mechanism-based trapping procedure to antibodies that were obtained using simple screening of hybridoma derived monoclonal antibodies. The best catalyst identified in the latter, from a set of 22 antibodies, displayed a $K_M = 330$ μmol dm^{-3} and a $k_{cat} = 10^{-5}$ min^{-1} which represents a rate acceleration (k_{cat}/k_{uncat}) of only 10^2. By selecting directly for catalytic activity rather then simple binding, this approach has been successful in obtaining catalysts that are capable of significantly greater rate accelerations than those obtained using conventional methods.

Gao *et al.*[27] used a similar approach in an attempt to obtain antibodies capable of amide hydrolysis using an α-amino boronic acid hapten as the selecting agent (Scheme 17). The authors suggested two mechanisms by which the boronic acid functionality could select antibodies for acyl transfer. Firstly, the hydration of the trigonal boronic acid to form the tetrahedral hydrate would mimic the transition state of the addition of water to the amide carbonyl. Antibodies which bind this transition state analogue could therefore catalyse hydrolysis by the conventional route of transition state stabilisation. Secondly, it was proposed that the Lewis-acidic hapten could select antibodies which contain complementary Lewis-basic residues within their binding pockets. The selection of such functionality could produce hydrolytic catalysts which operate by a nucleophilic mechanism.

A combinatorial antibody Fab library containing *ca.* 2×10^8 members was generated from a mouse immunised with a KLH conjugate of the boronic acid hapten. This was panned *in vitro* against a BSA conjugate of the hapten. Highly stringent washing with 8 mol dm^{-3} urea was carried out to remove non-binding and weakly binding phage, and the tight binders were then eluted with an acid wash at pH 2.2. Following four rounds of panning selection, 30 randomly selected clones were analysed and a single catalytic Fab was identified. Kinetic studies on the soluble Fab BL25 showed that this antibody catalysed the stereospecific hydrolysis of the L-proline isomer with $k_{cat} = 0.003$ min^{-1}, $K_M = 150$ μmol dm^{-3} and $k_{cat}/k_{uncat} = 4 \times 10^4$. A rate enhancement of this magnitude reduces the half-life of

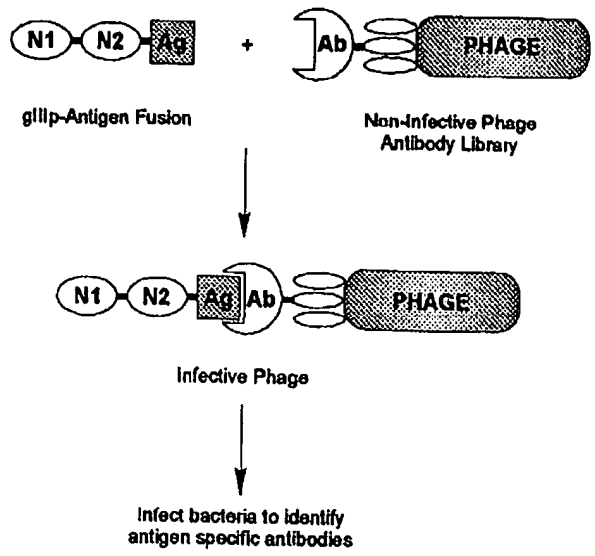


Fig. 7 The principles of Selectively Infective Phage (SIP) selection of antigen binding antibodies.

the primary amide from 17.5 years to 3.9 hours. This is an improvement of more than 2 orders of magnitude on the rate acceleration achieved by an antibody obtained using a phosphonate transition state analogue in a previous study. In addition, analysis of a panel of monoclonal antibodies generated against the hapten using hybridoma methodology displayed no hydrolytic activity against either proline carboxamide or methyl ester substrates. This is unexpected as the authors go on to suggest that the mechanism by which the hapten elicited catalytic activity was through simple transition state stabilisation, and not through the formation of a covalent interaction, as was initially proposed. If this is the case, then it would be expected that catalysts would have been obtained using the conventional hybridoma approach. The authors suggest that this is due to the fact that only a small fraction of the whole immune repertoire can be analysed using hybridomas. This work therefore demonstrates the effectiveness of a direct selection strategy in obtaining catalysts, when conventional approaches fail.

4.5 Selectively infective phage (SIP)

In a related study Gao *et al.*[140] adapted the Selectively Infective Phage (SIP) strategy[141] of phage-antibody selection in order to select directly for acyl transfer antibodies which operate by a covalent mechanism. SIP itself is a modification of conventional antibody phage display, in which phage infectivity is directly dependent on the formation of an antibody–antigen interaction (Fig. 7). The phage used in SIP selection are first rendered non-infective by deleting the *N*-terminal region of the gIII protein. The deleted region of the gIII protein is then produced separately fused to the antigen in question. The infectivity of the phage particle is then selectively restored by interaction between antibody fragments displayed on the surface of the non-infective phage, and the antigen fused with gIII protein fragment. Phage replication is therefore directly linked with antibody–antigen binding.

Gao *et al.*[140] devised a strategy based on SIP, whereby the restoration of infectivity could be linked with catalytic activity rather than binding (Fig. 8). Bi-functional probe compounds were designed which contained a reactive group at one end capable of forming a covalent link with catalytic residues in the antibody, and a labelling group at the other end which allowed attachment to the gIII protein fragment. The formation of a link between the phage particle and the gIII protein was then reliant on the catalytic activity of the antibody. The screening of antibody libraries using this system is then simply a

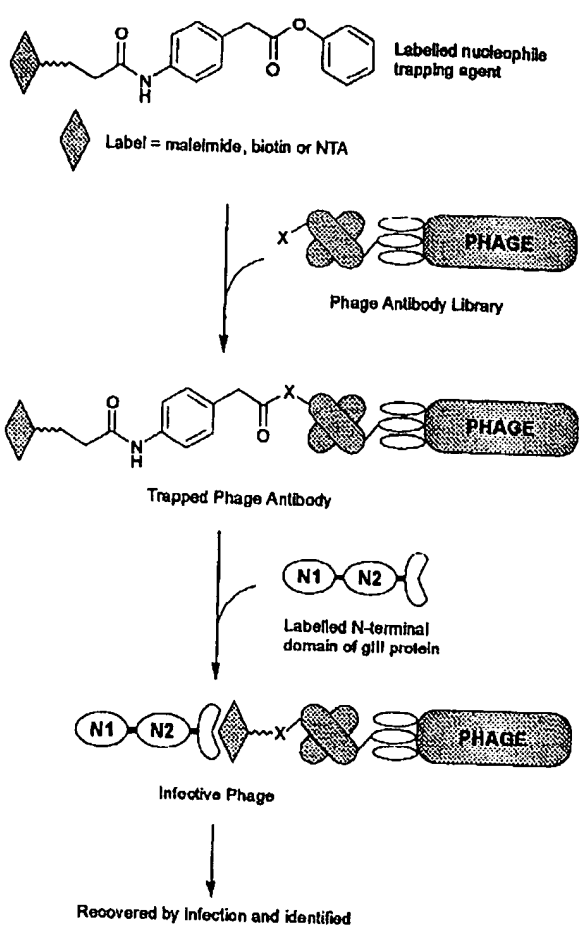


Fig. 8 Selection of nucleophilic catalysts using a labelled trapping agent to restore phage infectivity.

matter of retaining those phage which can successfully infect a bacterial host.

To demonstrate this strategy, the authors used two previously identified catalytic antibodies, PCP21H3[142] which was known to hydrolyse phenyl esters by a covalent mechanism, and PCP2H6[143] which catalyses the same reaction but does not use a covalent mechanism. Both antibodies were produced as non-infective phage-scFv's, and it was shown that the infectivity of the phage could be successfully restored in the case of PCP21H3-phage, but not in the case of PCP2H6-phage. This restoration of infectivity could be inhibited by the original phosphonate hapten used to generate both antibodies, demonstrating the specificity of the selection. Having demonstrated the viability of this method, the authors then went on to demonstrate its application to the selection of an active catalyst from a large library. The catalyst PCP21H3 was mixed with PCP2H6 in a phage ratio of up to $1:10^5$. A single round of selection was then carried out and it was found that the 60% of the recovered phage in the 10^5 library contained the PCP21H3 antibody. This represents an enrichment factor (% in selected/% in library) of 6×10^4. This method of selection should therefore offer an effective method of identifying active catalysts from large combinatorial antibody libraries.

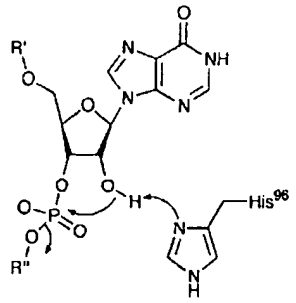
5 Engineering antibody catalysts

5.1 Introducing catalytic activity by site-directed mutagenesis

The conventional route to the creation of catalytic antibodies involves immunisation with a transition state analogue to obtain an antibody binding pocket which is complementary to a reaction transition state. Fletcher *et al.*[73] have employed a different approach, using protein engineering techniques to

introduce catalytic activity into a non-catalytic antibody. Starting with an antibody that binds specifically to the substrate, site-directed mutagenesis was used to change a single amino acid in the binding pocket. This single amino acid mutation was successful in producing an active catalyst.

This strategy was used to obtain a ribonuclease catalytic antibody starting from the RNA binding antibody Jel 103.[114] Based on the X-ray crystal structure of the antibody Fab fragment, in complex with inosine diphosphate, the authors targeted Arg^{H96} for mutagenesis, due to its close proximity to the 2′-OH of the ribose sugar. They hypothesised that the replacement of arginine for histidine at this position would result in activation of the 2′-OH of the sugar, leading to intramolecular cleavage of the phosphodiester back-bone in a similar fashion to that employed by ribonuclease enzymes (Scheme 43).[144]



Scheme 43 Proposed mechanism of action of ribonuclease antibody mutant HisH96 scFv, showing the key histidine residue.

Site-directed mutagenesis was carried out on a scFv fragment of Jel 103 to generate the mutant scFv (Arg^{H96}His). The ribonuclease activity of this scFv was investigated using fluorescence polarisation[145] with a fluorescein-labelled oligoriboinositide substrate. Effective hydrolysis of the RNA substrate was observed in the presence of the histidine mutant in a time-dependant fashion by monitoring the decline in the millipolarisation value of the RNA. This activity was confirmed by gel electrophoresis quantification of the free fluorescein-labelled oligo (rI) and by ethidium fluorescence quantification of the oligo(rI)·oligo(dC) duplex. The catalytic efficiency (k_{cat}/K_m) of the antibody was estimated to be 100 $dm^3\ mol^{-1}sec^{-1}$ by comparison with ribonuclease T_1 kinetics.

The authors have used the same approach to turn an antibody which binds the protein HPr into a hydrolytic antibody which cleaves the same protein.[74] In this case a quadruple mutant (EKHG) was found which had the best catalytic activity.

5.2 Intrabodies: intracellular catalytic antibodies

One interesting area of antibody engineering research, which has only recently been applied to catalytic antibody technology, is the adaptation of antibodies for intracellular expression. Antibodies are conventionally extracellular proteins due to the role they play in the humoral immune system. Only recently have functional antibody fragments been successfully expressed in the interior of a cell.[146] Such antibodies, referred to as Intrabodies, are finding use as high affinity ligands for analysis and as potential new therapies.

The major factor that has prevented effective intracellular expression of antibodies is the presence of a structurally essential disulfide bond in each antibody domain. Within the reducing environment of the cell cytoplasm, this bond is prevented from forming, and the resulting loss in stability prevents the antibody from folding into an active form. Insoluble, unfolded protein aggregates are then generally formed. Ohage *et al.* have overcome this problem by introducing stabilising mutations into the framework regions of a consensus V_L domain sequence. This hyperstabilised domain can be successfully expressed to high levels in the cytoplasm of *E. coli* as an F_V heterodimer, conveying stability onto its partner V_H domain.[147]

Ohage *et al.* took this approach a step further to produce the first catalytic intrabody.[147] Using their hyperstable V_L domain and the V_H domain from the anti-*para*-dinitrophenyl antibody B1-8 as a scaffold, they introduced the CDR loop sequences taken from the hydrolytic antibody 17E834 using the process of loop grafting. The resulting F_V heterodimer exhibited both the intracellular folding stability of the framework scaffold and the catalytic activity of 17E8. The F_V construct was produced in an *E. coli* host by cytoplasmic expression, and the resulting protein was found to fold reversibly under reducing conditions (0.5 mmol dm^{-3} β-mercaptoethanol). More significantly, the antibody fragment was found to exhibit hydrolytic activity using the *N*-formylmethionine phenylester substrate, with a K_M value of 126 μmol dm^{-3} and a k_{cat} of 1.6 min^{-1}. This compares favourably with the oxidised fragment with intact disulfide bridges, which displayed a K_M value of 820 μmol dm^{-3} and a k_{cat} of 1.9 min^{-1}. The authors go on to suggest that this first example of the production of an intracellular abzyme may open the way for the application of catalytic antibodies in the manipulation of cellular metabolism, an area which has, until now, been beyond the scope of antibody catalysis.

6 Applications of antibody catalysis

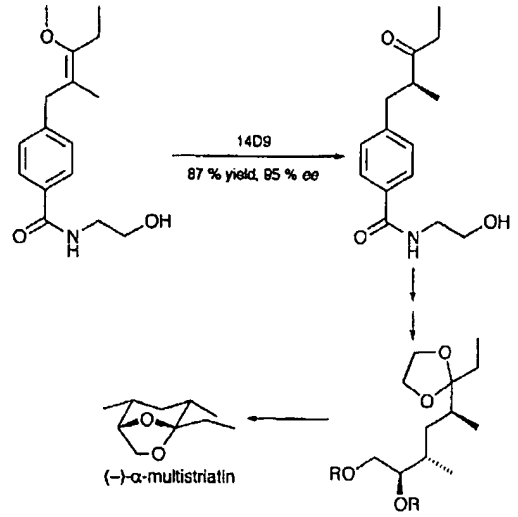
6.1 The first commercially available catalytic antibody

The aldolase antibody 38C2 has been made commercially available (Aldrich cat. no. 47,995-0; 48,157-2). This antibody has a broad substrate specificity, and similar catalytic efficiency to natural aldolases (Scheme 26). It has already been used in the preparation of key intermediates in a variety of natural products syntheses as described below.

6.2 Stereoselective natural product synthesis

The use of catalytic antibodies in synthesis has been discussed in detail in two recent reviews[148,149] and hence only key reactions will be described here.

In 1995 14D9, an antibody which catalysed enantioselective enol ether cleavage was used in the synthesis of the aggregation pheromone (−)-α-multistriatin. The antibody selectively cleaved the Z-enol ether to the (*S*)-α-methyl ketone in 95% ee This was then taken on in twelve steps to (−)-α-multistriatin (Scheme 44).[150]



Scheme 44

The aldolase antibody 38C2 has been used to produce two key (+)-*syn*-β-hydroxyketones used in the synthesis of the anticancer agents epothilone A and C through kinetic resolution

reactions (Scheme 45). In the first case the antibody was used to catalyse a retro-aldol condensation degrading the unwanted enantiomer and leaving the required (+)-*syn* product in 96% ee at 40% conversion, whilst the antibody was also used to catalyse the aldol condensation of an α,β-unsaturated aldehyde to its corresponding β-hydroxyketone in 75% ee at 10% conversion.[151]

The same antibody, 38C2, has been used to catalyse the enantioselective formation of the (*S*)-(+)-Wieland–Miescher ketone through a Robinson annulation reaction with >95% ee[152] This compound has been used as a key intermediate in the synthesis of a number of steroids. In a third reaction catalysed by the same antibody, 38C2, 1-deoxy-L-xylulose has been produced in two steps by the aldol addition of hydroxyacetone to an aldehyde.[51]

Two examples of synthetically useful stereo- and regioselective antibody hydrolyses have also been reported. Fujii and *et al.*[153] took antibody, 17E11 which selectively hydrolysed C-4 acylated 6-deoxy-6-fluoromonosaccharides showing no selectivity between the α- or β-anomers, or of the configuration of the C-2 substituent. To convert this antibody into one which would accept monosaccharides with a protected 6-hydroxy group they created a phage-display library of 17E11 and identified mutants which bound a modified hapten. This mutant was able to catalyse the hydrolysis of the C-4 derivative with 12-fold higher activity than the parent antibody.

Janda and co-workers have produced antibodies which are capable of selectively hydrolysing (+)-(*S*)-naproxen *p*-methylsulfonylphenyl ester in the presence of its enantiomer.[30] Using a reactive immunisation approach a catalyst capable of 90% ee at 26% conversion was produced. In order to improve on this approach they used a transition-state analogue and produced an antibody with a million-fold rate acceleration and 98% ee at 20% conversion. However, this antibody displayed severe product inhibition from the phenolic product and hence will not displace the current industrial resolution route which uses diastereomeric crystallisation.

6.3 Medical applications

A number of companies including Med-Immune (Gaithersburg, MD), Advanced Biotech Ltd (Ariel, Israel) and Prolifaron (San Diego, CA) are developing catalytic antibodies for use as therapeutics. These focus on two main applications, the activation and targeting of cytotoxic prodrugs in a modification of Antibody-Directed Enzyme Prodrug Therapy (ADEPT), and in the neutralisation of cocaine and other addictive narcotics. Other areas of interest include the neutralisation of the endotoxin LPS which is responsible for around 50% of deaths from Gram-negative sepsis infections.[154]

6.4 Prodrug activation

The previous review discussed the early research into the development of antibodies capable of activating specific prodrugs for incorporation into Antibody-Directed Abzyme Prodrug Therapy (ADAPT) systems. More recently Shabat *et al.*[155] have demonstrated that aldolase antibody 38C2 is able to catalyse a tandem retro-aldol–retro-Michael reaction on both prodoxorubicin and procamptothecin substrates to release the unmodified drug which can then inhibit topoisomerases I and II (Scheme 46). The *in vitro* activities of these drug activation systems were tested against HT29, LIM1215 and LNCap cells demonstrating that cell growth was significantly slower when antibody 38C2 was added together with the prodrug. The normally aldolase activity of 38C2 *in vivo* in mice injected with the antibody was found to persist for more than 3 weeks post-injection demonstrating that this catalytic activity is not neutralised or cleared more rapidly than other antibodies.

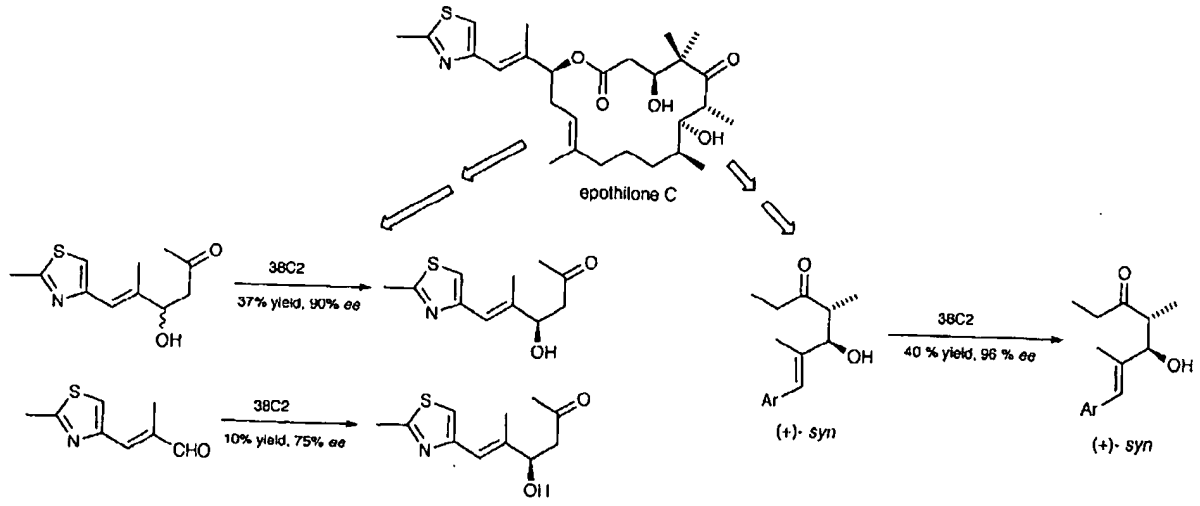
Taylor and co-workers have recently reported another antibody capable of hydrolysing carbamates,[156] and have also devised some suitable carbamate protected prodrugs,[157] but have yet to combine the two.

6.5 Anti-cocaine catalytic antibodies

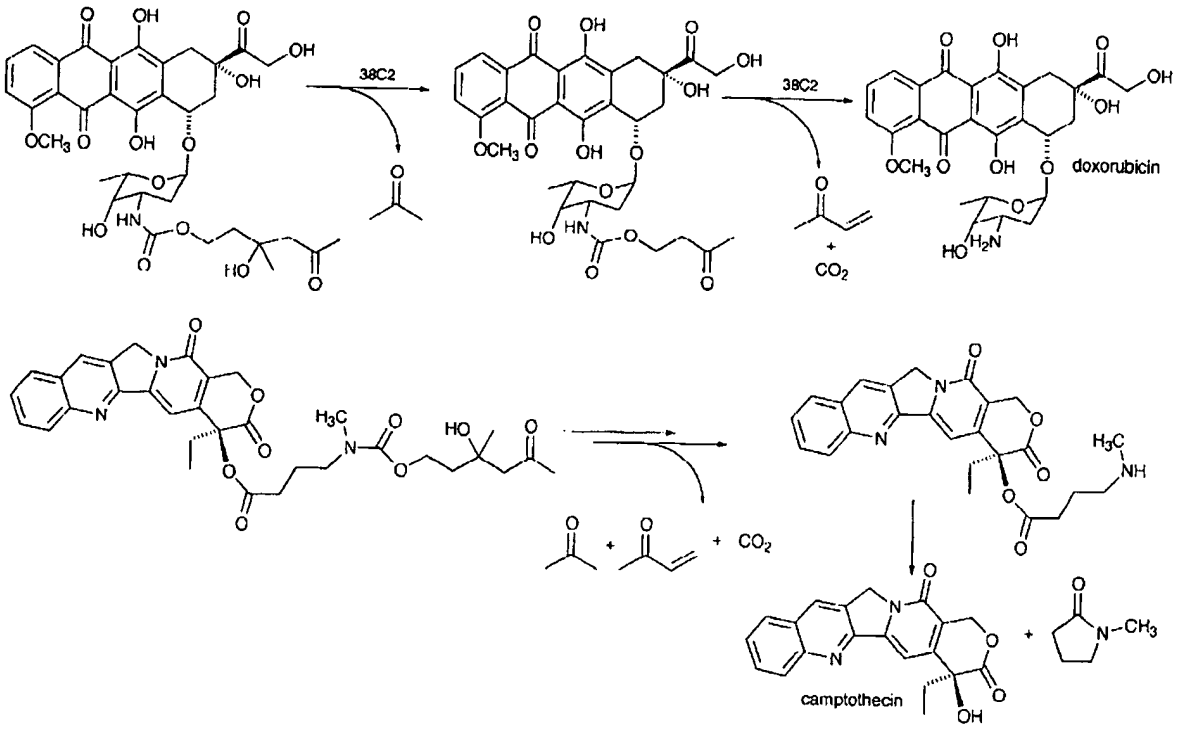
Several groups have continued working on the development of cocaine hydrolysing antibodies capable of degrading cocaine before it reaches the central nervous system. A number of new haptens have been prepared,[158] the transition-states for alkaline hydrolysis have been predicted by computer modelling[159] and further *in vivo* studies have shown that a reasonably efficient cocaine hydrolysing antibody 15A10 (Scheme 47) can protect rats who have overdosed on cocaine from seizures and sudden death.[160]

7 Comparison with other biomimetic catalysts

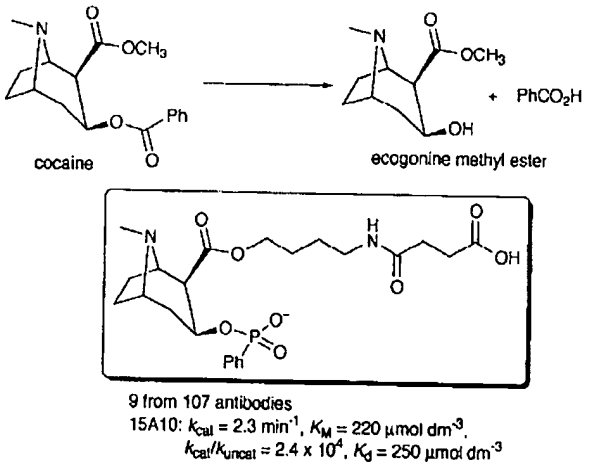
Since the last review a number of other approaches for creating biomimetic catalysts have been developed or explored further. Here we restrict comparisons to materials that have been selected in the same manner as catalytic antibodies, using transition-state analogues, or mechanism-based inhibitors, or designed specifically to stabilise transition-states. Catalytic cavities based on peptide, nucleotide, organic polymeric or small molecule receptors are included. A comparison of these catalytic activities with the intrinsic activity of albumins and other non-catalytic proteins is also presented. The areas of directed enzyme evolution,[161] enzyme engineering[162] and catalytic zeolites (zeozymes) and other minerals,[163] although pertinent, are beyond the scope of this review.



Scheme 45



Scheme 46



Scheme 47

7.1 Albumins as catalysts

It has been known for a number of years that serum albumins have the intrinsic ability to catalyse a wide range of less demanding chemical reactions,[164] and a summary of the best characterised examples are given in Table 4. These range from proton transfers (Kemp elimination/Meisenheimer complex decomposition),[165–168] racemisations/epimerisations,[171–173] spiro-pyran ring opening,[174] elimination[175] and ester/amide/carbonate hydrolysis.[169,170,176–180] In many of these cases, the presence of one or more lysines within a hydrophobic binding pocket in subdomain IIA of BSA which can be derivatised with pyridoxal 5'-phosphate (PLP) has been implicated as being responsible for the reaction. Pre-treatment of BSA with PLP destroys the hydrolytic activity reported above.[181] Colonna *et al.* have explored the effect of bovine serum albumin on a variety of reductions and Diels–Alder reactions as summarised in Table 4 and have found good asymmetric induction in a number of cases.[182–185] The structural and mechanistic reasons for this are currently unclear. It is useful to compare the rate accelerations produced with serum albumins as they can be considered as primitive enzyme benchmarks against which the success of other systems can be compared.

7.2 Molecularly imprinted (crosslinked) polymers

Through the research of Wulff and Mosbach, molecularly imprinted polymers (MIPs) based on highly crosslinked polystyrene, poly(methyl methacrylate) or other synthetic polymers have been promoted for use in chromatography as 'plastic antibodies'.[186] These polymers can be thought of as chemically and mechanically robust polyclonal antibody analogues which exhibit similar powers of molecular recognition to those of antibodies in both non-aqueous and aqueous conditions. Recent research on MIPs has focused on their potential use as catalysts.[187] The use of both transition-state analogue and 'bait & switch' imprint molecules has been investigated. The catalytic polymers reported to date are summarised in Table 5. A number of reactions for which catalytic antibodies have been produced have also been demonstrated with MIPs including the Aldol condensation,[188] Kemp elimination,[189] Diels–Alder cycloaddition,[190] β-elimination,[191,192] and ester hydrolysis.[193] As can be seen from the table, the rate accelerations produced by these polymers have been modest. This is thought to be due to the inflexibility of the highly crosslinked polymers and the slow rates of substrate binding–product debinding which are often the rate determining steps.

An alternative approach has been the bio-imprinting of proteins pioneered by Klibanov.[194] This involves lyophilisation of the protein in the presence of a template molecule chosen to change the binding site architecture of the protein so that an enzyme such as chymotrypsin can change its substrate specificity to favour peptides involving D-amino acids,[195] horse liver alcohol dehydrogenase can be made to accept NADP$^+$,[196] or in the case of albumins to induce catalytically active binding sites. These bio-imprinted proteins are then taken up in organic solvents and the template molecules extracted to leave the perturbed binding sites. If taken up in water the binding site changes are lost; however, Peißker and Fischer have recently reported that the imprinted properties of imprinted proteins can be retained in water if they are crosslinked prior to exposure to aqueous conditions.[197] They have termed these

Table 4 Albumin catalysts

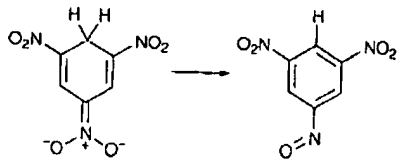
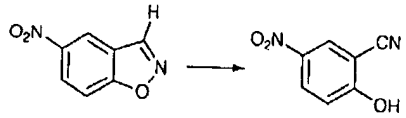
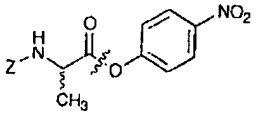
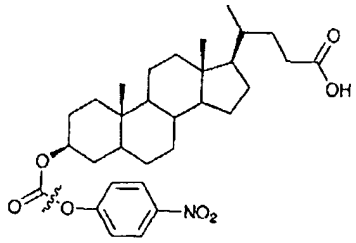
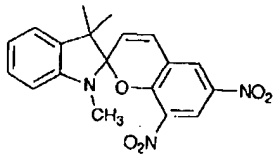
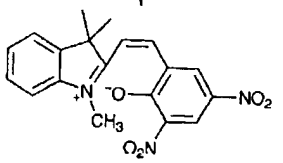
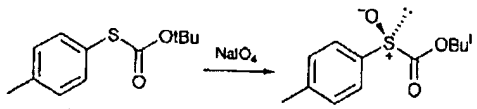
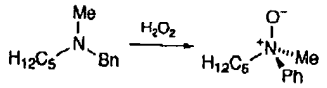
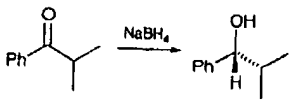
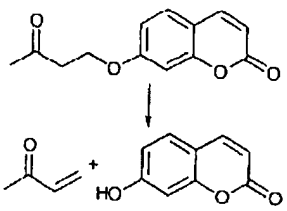
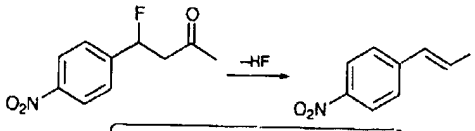
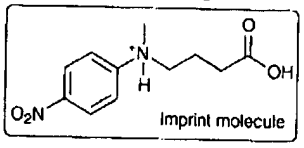
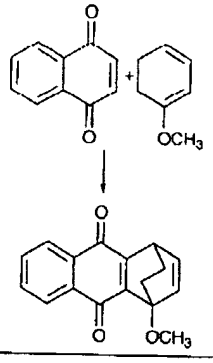
Reaction	Substrate	Source	Reaction conditions	k_{cat}	K_M/ mmol dm^{-3}	k_{cat}/k_{uncat}	Ref.
Meisenheimer complex decomposition		BSA	Phosphate buffer, pH 8.0	0.52 s^{-1}	ND	7.2×10^3	165
Kemp elimination		HSA	AMPSO buffer, pH 9.0	29 min^{-1}	3.1	5×10^3	166
		BSA		15 min^{-1}	0.7	2.5×10^3	167, 168
Ester hydrolysis		BSA	Bis-Tris buffer, pH 7.3	2.11 s^{-1} (D-Ala) 0.795 s^{-1} (L-Ala)	0.019 12	63×10^3	169
Carbonate hydrolysis		BSA	Tris buffer, pH 9.0 35 °C	7.8×10^{-2} s^{-1}	0.014	ND	170
Spiropyran opening		BSA	Phosphate buffer, pH 7.4	3.0×10^{-3} s^{-1}	~0.01	190	171
		HSA		2.0×10^{-3} s^{-1}		130	
		RSA		0.3×10^{-3} s^{-1}		22	
							
Sulfide oxidation		BSA	Borate buffer	ND	ND	ND 69.3% ee 60% yield	182
Tertiary amine oxidation		BSA	Borate buffer, pH 9.0	ND	ND	ND 66.8% ee 100% yield	183
Ketone reduction		BSA	Borate buffer, pH 9.2	ND	ND	ND 66% ee	184
β-Elimination		BSA	H_2O, pH 8.8	0.46 min^{-1}	0.15	390	175

Table 4 (*Contd.*)

Ref.	Reaction	Substrate	Source	Reaction conditions	k_{cat}	K_M/ mmol dm^{-3}	k_{cat}/k_{uncat}	Ref.
165	β-Elimination		BSA	H_2O, pH 7.0	8.9 min^{-1}	0.16	4240	196
			BSA (imprinted)	Ethyl acetate	267 min^{-1}	189	$k_{imp}/k_{non\text{-}imp}$ = 3.3	197
166 167 168			β-Lacto-globulin (imprinted)	CH_3CN, 39 °C	3.6×10^{-2} min^{-1}	28	9000	199
169	Diels–Alder		BSA	H_2O	ND	ND	ND 38% ee	185

proteins 'CLIPs' – crosslinked imprinted proteins. Most relevant to this review is the imprinting of bovine serum albumin with a transition state analogue to induce catalytic activity (HF elimination) (Table 4).[198,199] Slade and Vulfson found that BSA catalyses the elimination of HF from 4-fluoro-4-(*p*-nitrophenyl)butan-2-one at a rate approaching that of the antibody reported to catalyse the same reaction under aqueous conditions.[198] Ohya *et al.* found that imprinting BSA gave a 3-fold rate enhancement for the elimination when conducted in ethyl acetate.[199] Slade and Vulfson imprinted β-lactoglobulin with *N*-isopropyl-4-nitrobenzylamine which did not catalyse the elimination in its natural state. They found that this imprinted globulin catalysed the elimination reaction with a rate enhancement of 9000 in acetonitrile, when compared to the absence of the protein and around 3-fold that of the non-imprinted β-lactoglobulin.

In a more recent study Liu *et al.*[200] have reported that modifying egg albumin with *N*,*S*-bis(2,4-dinitrophenyl)glutathione and then treating it with phenylmethanesulfonyl fluoride and sodium hydrogen selenide produced a protein with glutathione peroxidase activity.

In general the molecular imprinting approach has yielded less efficient catalysts than catalytic antibodies for similar reactions, but the approach is attractive due to its simplicity and speed.

7.3 Linear synthetic polymers (synzymes)

The catalytic properties of linear non-peptidic polymers have been explored by a number of groups. Early work in this area has been reviewed by Klotz[201] who termed these compounds 'synzymes'. A summary of recently reported synzymes is given in Table 6. Hollfelder and co-workers[202] have shown that polyethyleneimine (PEI, MW 25 kDa) could be 'tuned' by alkylation with different ratios of methyl iodide, benzyl bromide and dodecyl iodide to give a polymer with an increased number of microenvironments which accelerated the Kemp elimination reaction of 5-nitrobenzisoxazole. This is thought to be due to the lowering of the pK_a values of some of the polymer amines by up to 5 pH units, so improving their basicity. As the polymers have random primary structures, and hence are assumed to form a heterogeneous mixture of secondary and tertiary structures, precise details on the elements effecting catalysis are difficult to determine. Menger *et al.* have produced a phosphatase synzyme using poly(allylamine) (PAA, MW 50–60 kDa) polymers capable of hydrolysing bis-(*p*-nitrophenyl)-phosphate by modifying it with a variety of amino acid-like side-chains and adding iron(III)[203] and more recently a reducing catalyst by modifying either PEI or PAA and using this to reduce benzoylformic acid to mandelic acid in the presence of dihydropyridine and zinc, magnesium or copper.[204] Similarly Miller and Ford have produced a range of minimally crosslinked latex copolymers (25% mass vinylbenzyl chloride:73% alkyl methacrylic acid:1% divinylbenzene:1% (3,4-divinylbenzyl)trimethylammonium chloride) which were then modified with trimethylamine or tributylamine to introduce anionic binding sites. These were found to catalyse the hydrolysis of a variety of alkyl *p*-nitrophenyl esters.[205] Again, whilst these are cheap and simple to prepare, it is difficult to determine their exact mechanism of action due to the heterogeneity of the structures.

7.4 Nucleic acid catalysts

Altman and Cech's discovery of ribozymes predates that of the generation of catalytic antibodies by several years.[206,207] Whilst a major effort has been made into the elucidation of the structure and mechanism of naturally occurring RNA catalysts which are capable of a variety of phosphate ester chemistry several groups have been involved in trying to create nucleic acid structures capable of catalysing a range of other reactions as summarised in Table 7. This area is reviewed in more depth by Famulok and Jenne.[208] Several groups have selected nucleic acid aptamers (Latin *aptus* = to fit) that have reasonable affinity for a transition-state analogue and hence should be capable of catalysing a reaction in the same way as catalytic antibodies. Metal insertion,[209,210] biphenyl isomerisation[211] and ester hydrolysis[212] catalysts have all been produced using this approach. Overall these catalysts are less efficient than their antibody counterparts, probably due to their lower affinity

Table 5 Catalytic molecularly imprinted polymers

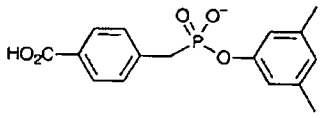
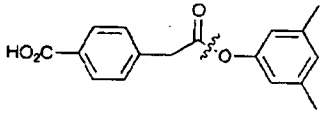
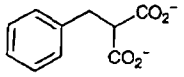
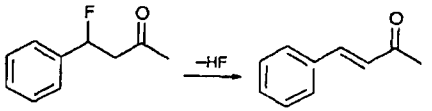
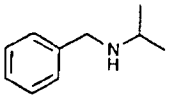
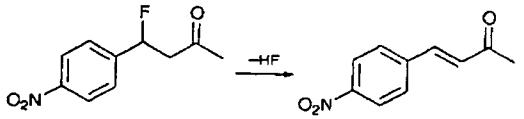
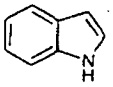
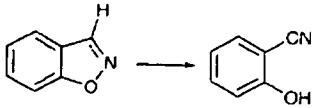
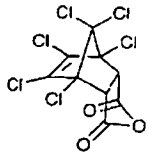
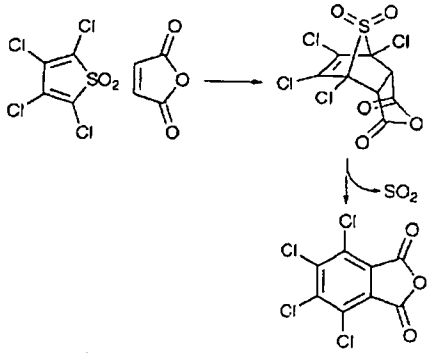
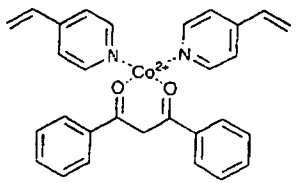
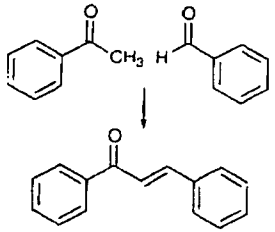
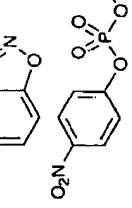
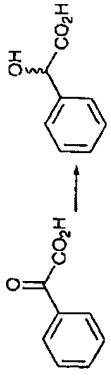
Reaction	Imprint molecule	Reaction	Polymer	Reaction cond.	Kinetic constants	Rate acceleration	Ref.
Ester hydrolysis			4-VP EGDMA	1:1 water: ethanol pH 7.6	$k_{cat} = 8 \times 10^3$ min^{-1} $K_M = 0.60$ mmol dm^{-3} $K_i = 0.025$ mmol dm^{-3}	$k_{cat}/k_{uncat} = 100$ $k_{imp}/k_{amidine} = 5$	193
HF elimination			2-AEMAA MAA EGDMA	benzene	$k_{cat} = 1 \times 10^{-2}$ min^{-1} $K_M = 27$ mmol dm^{-3}	$k_{imp}/k_{non\text{-}imp} = 7.5$ $k_{imp}/k_{amine} = 3.2$	191
			4-VP EGDMA	3:1 water: ethanol	$k_{cat} = 0.205$ min^{-1} $K_M = 0.484$ mmol dm^{-3} $K_d = 7.43$ mmol dm^{-3}	$k_{imp}/k_{non\text{-}imp} = 7.2$ $k_{imp}/k_{pyridine} = 4 \times 10^4$	192
Kemp elimination			MAA EGDMA	dichloro-methane	$k_{cat} = 7.9 \times 10^{-4}$ min^{-1}	$k_{imp}/k_{non\text{-}imp} = 2.4$	189
Diels–Alder cycloaddition			MMA EGDMA	82 °C acetonitrile	$appk_{cat} = 3.82 \times 10^{-2}$ min^{-1} $K_M = 42.5$ mmol dm^{-3} $K_d = 5.98$ mmol dm^{-3} EM = 128 mol dm^{-3}	$(k_{imp}/k_{non\text{-}imp})/k_{uncat} = 270$ (no turnover)	190
Aldol condensation			4-VP styrene DVB	100 °C DMF	$V_{max} = 0.61$ μmol h^{-1} $K_M = 1.23$ μmol dm^{-3} $K_i = 60$ μmol dm^{-3}	ND	188

Table 6 Synzymes

Reaction	Polymer	Modification	Substrate	Reaction conditions	k_{cat}/min^{-1}	K_M/mmol dm^{-3}	k_{cat}/k_{uncat}	Ref.
Kemp elimination	PEI 7.D/2.2	Dodecyl iodide (0.23 eq.) Benzyl bromide (0.26 eq.) Methyl iodide (02 eq.)		H_2O, pH 5.86	40 (>5 per site)	4.2	$>1.0 \times 10^6$ EM = 5055	202
Phosphate	PAA	*p*-Hydroxybenzoic acid (10%) Imidazole-4-acetic acid (15%) *n*-Octanoic acid (7.5%) Fe^{3+} (5%)		Phosphate buffer, pH 7.00	2.5×10^{-5}	ND	31400	203
Reduction	PAA	Dihydropyridine (10%) 2-Mercaptopropionic acid (10%) Imidazole-4-acetic acid (10%) *n*-Hexanoic acid (2.5%) 1-Naphthoic acid (10%) Zn^{2+} (10%)		Tris buffer, pH 7.2	ND	ND	(42% of dihydropyridines activated)	204

and limited functionalisation. Attempts to produce Diels–Alderase[213] or transesterase[214] aptamers using transition-state analogues have also failed to date. These indirect approaches in which nucleic acid libraries are screened for their binding affinity rather than directly for catalytic ability have proved less successful than the cases where catalysis has been selected for directly. Hence Tarasow *et al.*[215] and Scelig and Jäschke,[216] have both selected Diels–Alderase RNA molecules using the fact that the formation of the Diels–Alder adduct could be used to biotinylate active RNA molecules. Other activities that have been detected using this direct selection approach are amide bond formation (*cf.* ribosomal RNA activity)[217,218] and alkylation reactions.[219] All of the latter examples are not true catalysts in that the RNA itself becomes modified and so turnover cannot occur. However, these systems are useful from the viewpoint of the requirement of such activities in an 'RNA world' as a precursor to life on earth. One other area of recent interest has been the successful incorporation of modified nucleotides in Systematic Evolution of Ligands by Exponential Enrichment (SELEX) experiments allowing the functionality present in the nucleotide libraries to rival that of the proteinogenic amino acids. Hence Sakthivel and Barbas have shown that a C5-imidazole functionalised deoxyuridine is tolerated in place of thymidine as a substrate for reverse transcriptases and DNA polymerases. They have shown that incorporation of this modified base allows the selection of a zinc dependent RNA cleaving DNA enzyme.[220] In an alternative approach it has been shown that certain nucleotide catalysts could recruit non-metal cofactors to facilitate phosphodiester cleavage. These include a DNA polymer, which requires L-histidine to cleave RNA phosphodiester bonds.[221] Earlier research into the recruitment of cofactors/coenzymes has been reviewed by Joyce.[222]

7.5 Peptide catalysts

The use of small peptides (MW < 5000) as catalysts had not been extensively explored at the time of the last review, the seminal paper being Benner's work on a 14-residue α-helical peptide which catalysed the decarboxylation of oxaloacetate.[223] However as our understanding of peptide folding has improved a number of small stable protein domains and *de novo* designed sequences have been identified through the work of Imperiali,[224] Degrado[225] and others. Baltzer and Broo have conducted a comprehensive study on the structure and activity of a variety of 42 residue helix-loop-helix peptides.[226] They have used this stable motif as a platform for a number of different chemical activities as summarised in Table 8.[227–230] These peptides demonstrate that small peptides are capable of functioning as catalysts, however they do not possess a sufficiently well developed binding pocket to allow substrate recognition, or in many cases turnover. Imperiali and co-workers have also produced a peptide modified with a pyridoxamine unit for use as an aminotransferase.[231]

The most interesting peptide based catalyst is that reported by Ghadiri and co-workers who have developed a 'peptide ligase' capable of catalysing the formation of an internal amide bond in an activated precursor of the catalytic template.[232] Hence the system is capable of autocatalysis.

Distefano *et al.* have exploited the small adipocyte lipid binding protein (ALBP) composed of 131 amino acids which possesses an architecture of two orthogonal β-sheets as a scaffold for investigating a number of different catalytic activities including enantioselective reductive amination of α-keto acids to α-amino acids by introduction of a covalently bound pyridoxamine cofactor,[233] and the hydrolysis of amides and esters by the introduction of a phenanthroline ligand.[234] With ALBP-Phen-Cu(II) enantioselective hydrolysis of amino acid methyl or ethyl esters at rates 32–280-fold above background were observed at pH 6.1 with up to 7.6 turnovers. The enantiomeric excess of the products ranged was in the range 31–86%. ALBP-

Table 7 Nucleic acid catalysts

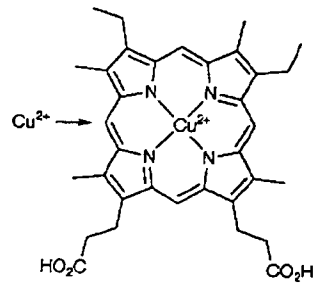
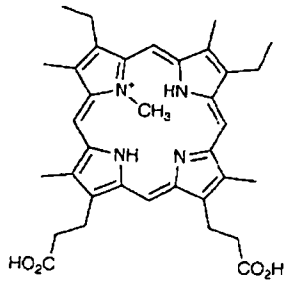
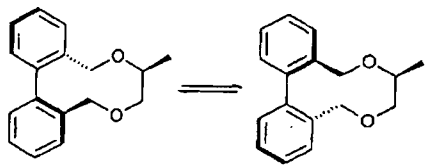
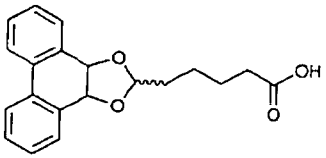
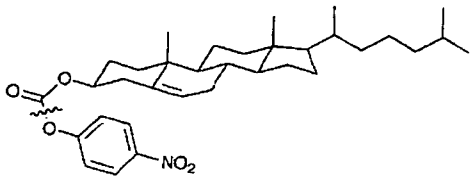
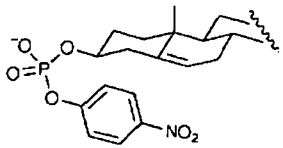
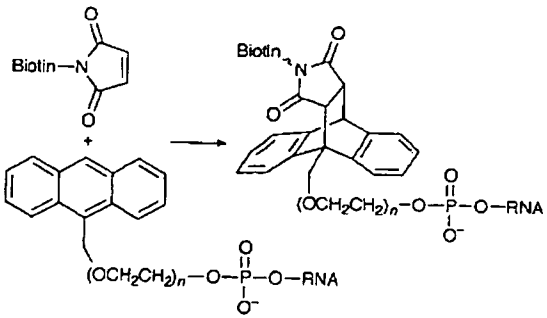
Reaction	Substrate	Template/TSA	Nucleic acid type and size	k_{cat}/min^{-1}	K_M/μmol dm^{-3}	K_I/μmol dm^{-3}	k_{cat}/k_{uncat}	Ref.
Metal insertion			RNA, 35-mer pH 8.0	0.92	14	0.120	460	20
			DNA, 33-mer pH 6.2	0.23	2300	3.6 (K_d)	1400	210
Isomerisation			RNA, 165-mer pH 5.75, 28 °C	2.8×10^{-5}	542	7	88	211
Ester hydrolysis			RNA, 110-mer pH 7.5	2.1×10^{-5}	29	10 (K_d)	110	212
Diels–Alder reaction			RNA, 49-mer	1.18	119 (dienophile)		Turnover with a 38-mer	216

Table 7 (*Contd.*)

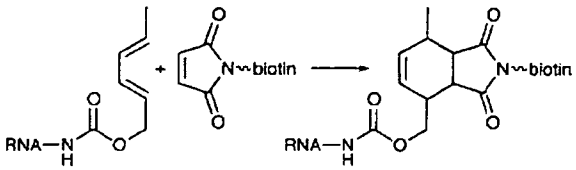
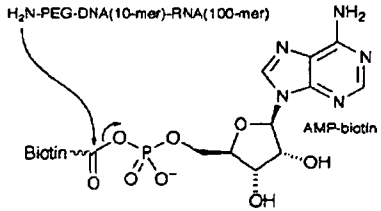
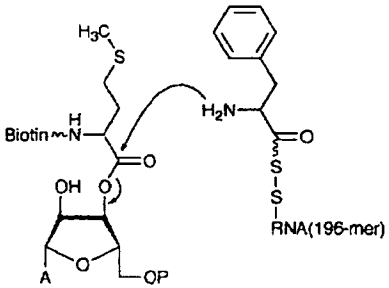
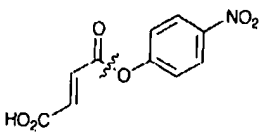
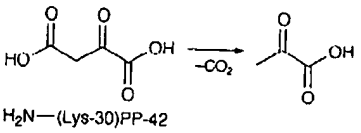
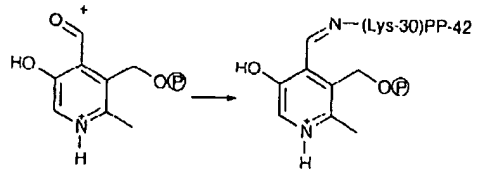
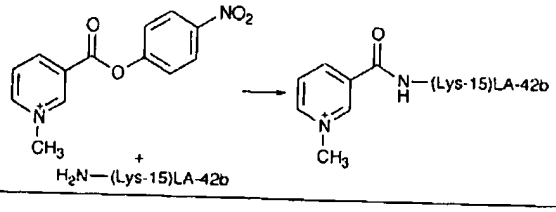
Reaction	Substrate	Template/TSA	Nucleic acid type and size	k_{cat}/min^{-1}	K_M/μmol dm^{-3}	K_I/μmol dm^{-3}	k_{cat}/k_{uncat}	Ref.
Diels–Alder reaction			RNA, 100-mer pH 7.0	0.66	2300 (dienophile)	32.5	2 mol dm^{-3} (EM) No Turnover	215
Amide formation		NA	RNA, 100-mer pH 7.0, 25 °C	0.04	2.3	NA	~10^5 No Turnover	217
Amide formation		NA	RNA, 196-mer pH 7.4, 25 °C	0.0	190	188	~10^6 No Turnover	218

Table 8 Catalytic peptides

Catalyst	Substrate	Reaction conditions	k_{cat}	k_{cat}/k_{uncat}	Ref.
KO-42		pH 4.1 Acyl transfer	0.10 dm^3 mol^{-1} s^{-1}	$k_{cat}/k_{4\text{-methylimidazole}}$ = 1140 No turnover	227
NP-42		pH 7.0, 25 °C Decarboxylation	0.015 dm^3 mol^{-1} s^{-1}	$k_{cat}/k_{Butylamine}$ = 10 No turnover	228
PP-42	H_2N—(Lys-30)PP-42 	pH 4.4, 25 °C Imine formation	ND	ND	229
LA-42	 H_2N—(Lys-15)LA-42b	pH 5.9, 25 °C Acyl transfer	2.75×10^{-2} dm^3 mol^{-1} s^{-1}	$k_{cat}/k_{4\text{-methylimidazole}} \sim 5$ No turnover	230

Phen-Cu(II) was also found to hydrolyse picolinic acid nitromethylanilide under similar conditions at a rate of 1.6×10^4-fold above background. These rates are comparable to those of other copper(II) complexes, but significantly lower than 'free' Cu(II). Distefano has improved the catalytic efficiency of both phenanthroline and pyridoxamine systems by using different tethering points within the binding site of the related protein, Intestinal Fatty Acid Binding Protein (IFABP), by introduction of cystine residues at positions 60, 72 and 104 in the sequence as alternatives to the naturally occurring Cys.[117]

7.6 Cyclodextrin, cyclophane and other small molecule receptor catalysts

Both naturally occurring (cyclodextrin) and synthetic (cyclophane) small molecule receptors have been decorated with suitable groups in order to turn them into biomimetic catalysts. Many of these systems are not true catalysts as turnover of substrate is not observed. However a number of recent systems have achieved this and these are summarised in Table 9.

Breslow has explored the catalytic activity of a wide variety of derivatised cyclodextrins over the past three decades.[235] Most recently he has identified a bipyridyl cyclodextrin dimer which is capable of increasing the rate of hydrolysis of a *tert*-butylbenzyl ester by 1350-fold over the background reaction and is also capable of turnover in this reaction in which alkoxide leaving is rate determining.[236]

Cyclodextrins have also been modified for use in catalysing the Kemp elimination of 5-nitrobenzisoxazole,[237] aldol condensation,[238] oxidative cleavage of the 15,15′-double bond of β,β-carotene,[239] and as a cytochrome P_{450} mimic.[240]

Tsutsumi *et al.* have used a cyclodextrin–peptide hybrid as a catalyst for the hydrolysis of *p*-nitrophenyl acetate.[241] The peptide used was a 19-mer composed primarily of alanines, but also incorporating two pairs of intramolecular salt bridges (Glu4–Arg8; Glu12–Arg16) and a histidine at position 10 and β-cyclodextrin appended to the side-chain of glutamate 14. It was found that substitution with an additional glutamic acid at position 6 in the peptide resulted in a compound which hydrolysed *p*-nitrophenyl acetate 2.25 faster, and both hybrids catalysed the hydrolysis reaction just under 10^3-fold faster than the background reaction.

Other groups have developed synthetic receptors to catalyse related reactions. Kennan and Whitlock have prepared a bis-naphthalene sandwich complex equipped with a dialkyl amino-pyridine which is capable of catalysing the Kemp elimination of 5-nitrobenzisoxazole[242] whilst Diederich has modified a cyclophane scaffold with flavin and thiazolium units to produce a pyruvate oxidase mimic which achieves turnover by electrochemical regeneration of the flavin.[243] Rebek and co-workers have produced a molecular capsule that catalyses the Diels–Alder reaction of 2,5-dimethylthiophene dioxide and *p*-benzoquinone,[244] and Sanders, a porphyrin trimer which accelerates the hetero-Diels–Alder reaction of pyridylbutadiene and 3-nitrosopyridine.[245] A self-replicating version of another Diels–Alder reaction has been reported by Wang and Sutherland.[246]

7.7 Conclusions and future directions

Catalytic antibodies have now been under development for around fifteen years. It has become apparent during this period that antibodies are capable of functioning as catalysts for a broad range of reactions including examples with no known biological counterpart. The regio- and stereoselectivity of antibody catalysed reactions has also been demonstrated to match that of enzymes. In these areas, catalytic antibodies are currently unrivalled by any of the other types of biomimetic catalyst described in this section. However, the absolute efficiency of catalytic antibodies is still several orders of magnitude lower than for comparable enzymes. In all but a few examples, such as the aldolase antibodies, the catalytic activity lies closer to that of albumin/simple peptide systems than to evolutionary optimised enzymes. Several groups have employed the full armoury of current protein engineering and protein-display technologies in order to optimise the activity of specific antibody catalysts; however, improvements beyond one or two orders of magnitude have not been observed. One major step forward in the last five years has been the recognition that the use of mechanism based inhibitors alone, or in combination with transition-state analogues, to generate new catalytic activities using 'reactive immunisation' has often produced better

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Table 9 Miscellaneous biomimetic catalysts

Reaction	Catalyst	Reaction conditions	k_{cat}	K_M	k_{cat}/k_{uncat}	Ref.
Kemp elimination		Bis-Tris, pH 7.35 H_2O:MeOH (9.1)	0.02 s^{-1}	ND	3670 (140 *cf.* amine)	237
Ester hydrolysis		HEPES, pH 7.0 30% DMSO	2.7×10^{-3} s^{-1}	6.0×10^{-4} mol dm^{-3}	1350	236
Aldol condensation		Phosphate, pH 8.00	2×10^{-5} mol dm^{-1}	ND	80	238

Table 9 (*Contd.*)

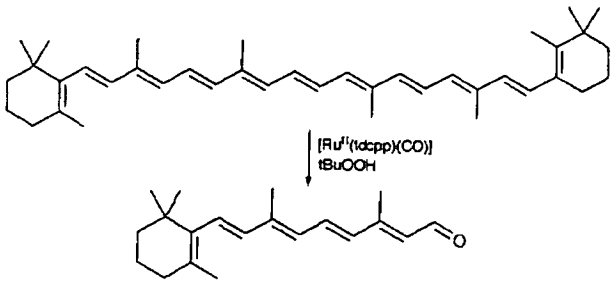
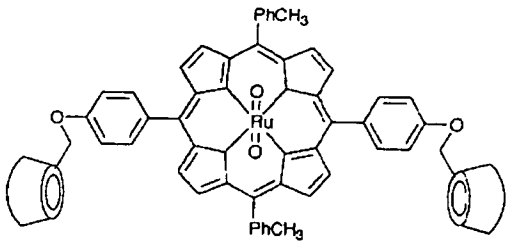
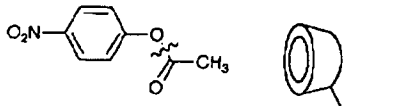
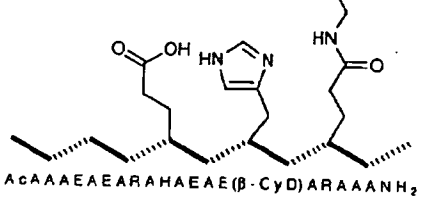
Reaction	Catalyst	Reaction conditions	k_{cat}	K_M	k_{cat}/k_{uncat}	Ref.
Oxidative alkene cleavage		H_2O–C_6H_{14}–$CHCl_3$	ND	ND	ND	239
						
Hydrolysis		Phosphate buffer, pH 6.0, 25 °C	$5.6 \times 10^{-3}\ s^{-1}$	9.9×10^{-4} mol dm^{-3}	829	241
						

Table 9 (*Contd.*)

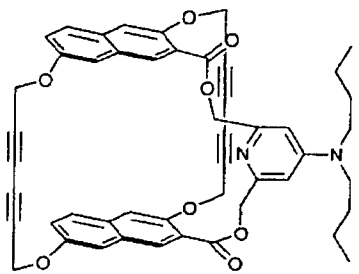
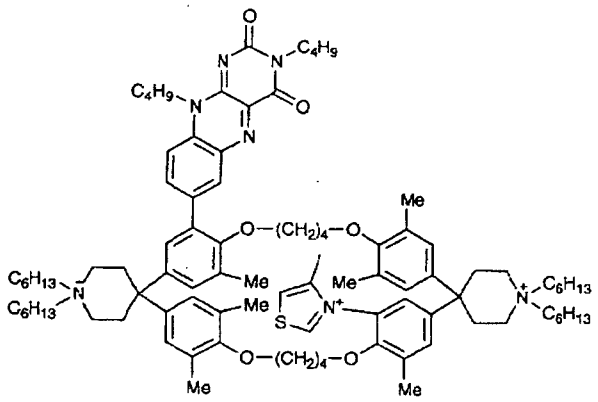
Reaction	Catalyst	Reaction conditions	k_{cat}	K_M	k_{cat}/k_{uncat}	Ref.
Kemp elimination		$CDCl_3$, RT	$6.3 \times 10^{-4}\ s^{-1}$	$K_a = 96\ dm^3\ mol^{-1}$	5869 (rel. to pyridine)	242
Oxidation		MeOH	$0.22\ s^{-1}$	21 mmol dm^{-3}	ND	243
						

Table 9 (*Contd.*)

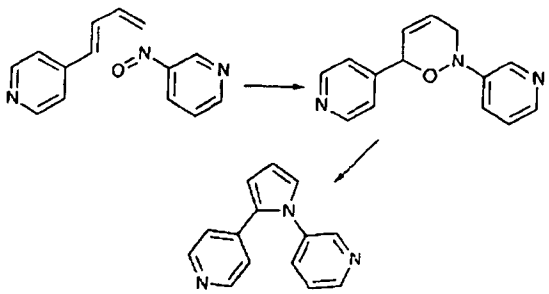
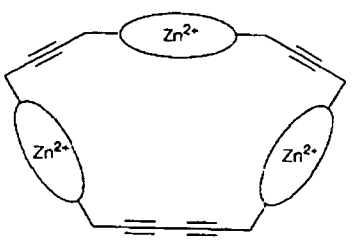
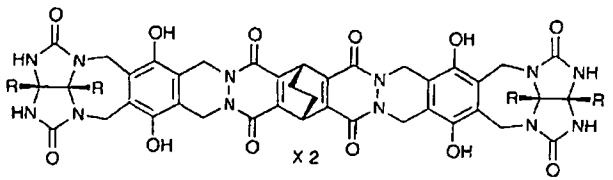
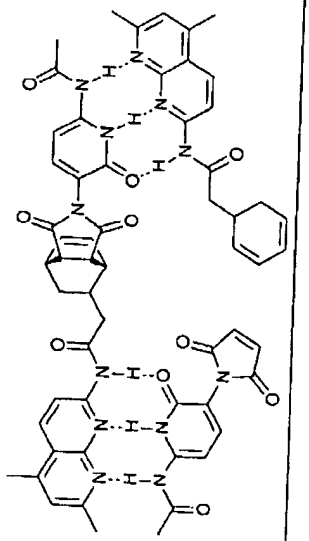
Reaction	Catalyst	Reaction conditions	k_{cat}	K_M	k_{cat}/k_{uncat}	Ref.
Hetero-Diels–Alder		CH_2Cl_2, 25 °C	4.0 M^{-1} s^{-1}	$K_b = 2.3 \times 10^8$ dm^3 mol^{-1}	1030	245
Diels–Alder		Xylene, 40 °C	ND	ND	ND	244
						

Table 9 (*Contd.*)

Reaction	Catalyst	Reaction conditions	k_{cat}	K_M	k_{cat}/k_{uncat}	Ref.
Self-replicating Diels–Alder reaction		CD_2Cl_2, 23 °C	7×10^{-3} $dm^3\ mol^{-1}\ s^{-1}$	228 mol dm^{-3}	E.M. = 4 mol dm^{-3}	246

catalysts. This has proved especially successful in the case of hydrolytic and aldolase catalytic antibodies. The limitations of the sole use of transition-state analogues has been discussed in detail in a review by Mader and Bartlett.[247] As with the search for new nucleic acid catalysts and enzymes modified by directed evolution, approaches involving direct screening or selection of libraries for catalytic activity has proved by far the most successful. The development of improved catalytic screening and selection methods is one area that will facilitate the identification of a wide variety of catalytic materials. The alternative approach of using site specific mutagenesis on antibodies with known high-resolution antibody–antigen structures has also proved successful in generating new DNA or protein hydrolytic antibodies in the limited number of cases studied,[73,114] and this approach should be explored further.

Currently the high costs of large-scale antibody production prohibit the use of catalytic antibodies in the preparation of fine chemicals on anything but a small laboratory scale. New methods of expression of antibody fragments in plants or other organisms are still not efficient enough to overcome this problem. However, due to their biocompatibility, the potential of catalytic anibodies in therapeutics such as drug delivery systems is more probable, as is the possibility of active immunisation of drug addicts.

Catalytic antibodies have also proved to be ideal model systems for testing out fundamental theories of enzyme catalysis. They offer a simplification of the complex interplay of factors such as desolvation, proximity, electrostatic stabilisation and acid/base catalysis that form the basis of enzyme chemistry. Because of the perturbed reaction profiles observed with antibodies it is generally easier to identify how specific contributions benefit catalysis, whereas the more balanced profile seen with most enzymes conceals a mixture of factors that are not easy to dissect.

There are several questions that need to be considered at this time, when proposing using antibodies as catalysts. Is the antibody framework of six hypervariable loops grafted on to a β-barrel an efficient framework for a catalytic protein? There are few, if any examples of enzymes using this type of structure as the foundation for a catalytic pocket. It has been suggested that 'mature' high affinity antibodies are too inflexible to allow for the possibility of the binding pocket to function in an induced fit manner, reorganising to bind several key conformations along the reaction co-ordinate as occurs in enzymes. Catalytic antibodies are currently thought to primarily act in a 'lock & key' fashion although evidence of multipleconformational changes is difficult to detect.[248] The use of alternative tertiary and quarternary folds is now possible through the phage display of other proteins such as knottins.[249] This will allow a number of alternative peptide folds to be explored for their predilection for catalysis.

The advent of phage display of antibody fragments in 1989 was anticipated to lead to the demise of the hybridoma technique for producing monoclonal antibody fragments. However, the routine preparation of antibody fragments of similarly high affinity to those obtained from the hybridoma method has still to be achieved using either naïve or immunised phage display libraries. Newer display techniques such as ribosome display,[250] and mRNA-protein fusions,[251] offer the possibility of creating larger libraries (10^{11}–10^{13} members), not limited by the transfection of competent cells. Artificial compartmentalisation of protein biosynthesis systems using mineral oil is an alternative method that has recently been reported.[252] All of these methods preserve the link between the genomic (DNA/RNA) and phenomic (protein) material of a library in a similar manner to the original phage display approach. They also all offer the possibility of selecting library members based on their ability to perform chemical reactions, and may supersede phage display in the long term. The use of antibody fragments is still hampered by the problem of economically producing large

quantities of a specific scFv in correctly folded form. Whilst several suitable human and murine framework regions which give good expression have been identified, the grafting on of different hypervariable loops on to these frameworks still leads to wide variations in levels of expression.[253] The incorporation of chaperone proteins in the protein expression systems has produced good results in a number of cases, but considerable work is still required to optimise the expression of individual antibody fragments.[254] Once this enabling technology has been perfected a wide variety of applications for catalytic antibodies can become commercially feasible.

8 Acknowledgements

Neil Thomas would like to thank the Royal Society for a University Research Fellowship and the EPSRC for a Ph.D. Studentship for James Stevenson.

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